Heparan sulphate glycosaminoglycans derived from endothelial cells and smooth muscle cells differentially modulate fibroblast growth factor-2 biological activity through fibroblast growth factor receptor-1

David BERRY*†‡, Zachary SHRIVER‡, Barbara NATKE‡, Chi-Pong KWAN‡, Ganesh VENKATARAMAN† and Ram SASISEKHARAN†‡§¹

*Harvard Medical School, 160 Longwood Avenue, Boston, MA 02215, U.S.A., †Harvard-Massachusetts Institute of Technology, Division of Health Sciences and Technology, 77 Massachusetts Avenue, Cambridge, MA 02139, U.S.A., ‡Biological Engineering Division, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139, U.S.A. and §Center for Biomedical Engineering, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139, U.S.A.

Fibroblast growth factor (FGF) signalling is involved in a wide range of important biological activities with differential effects in various cell types. The activity of FGF is modulated by heparin/heparan sulphate-like glycosaminoglycans (HSGAGs), found both in the extracellular matrix and on the cell surface. HSGAGs affect FGF signalling by interacting with both the growth factor and the FGF receptor (FGFR). In this study we sought to investigate whether HSGAGs at the cell surface of bovine aortic endothelial cells (BAEC) and smooth muscle cells (SMC) can differentially modulate FGF signalling in these cell types and modulate their differential response to FGF. We find that SMC and BAEC express the same FGFR isoforms and bind FGF2 with equal affinity at the cell surface, yet FGF has a markedly

higher proliferative effect on SMC than on BAEC. Isolated HSGAGs from these two cell types were found to elicit distinct patterns of proliferation in chlorate-treated cells. Furthermore, examination of focal sequences reveals that HSGAGs from SMC, but not those from BAEC, retain the sulphation pattern necessary to induce FGF2 activity. As such, the differences in FGF2-mediated proliferation can be explained by the distinct cell surface HSGAGs of the two cell types. We conclude that the focal sequences of cell surface HSGAGs from SMC and BAEC govern, at least in part, the differential activity of FGF2 on these two cell types.

Key words: atherosclerosis, extracellular matrix, heparin.

INTRODUCTION

Smooth muscle cells (SMC) and endothelial cells (EC) have been implicated in the pathophysiology of many disease states, including atherosclerosis. Key events in the induction of atherosclerosis include migration of SMC to the intima and subsequent proliferation [1]. Basic fibroblast growth factor (FGF) 2 is one of a spectrum of potent stimulatory molecules that act on SMC. FGF2 is typically stored extracellularly, bound to heparin/heparan sulphate-like glycosaminoglycans (HSGAGs). HSGAGs, the polymeric carbohydrate moiety of proteoglycans, are ubiquitous in the extracellular matrix (ECM) and on the cell surface [2]. HSGAGs in the ECM store FGF2 and prevent its degradation by proteolytic enzymes [3]. Alterations to the vascular endothelium are thought to induce the onset of pathological states such as atherosclerosis [4] through the release of mitogenic molecules, such as FGF2, from their ECM storage sites. Once released from the ECM, FGF2 is incorporated into a ternary complex by binding to its high affinity protein kinase receptor and low affinity HSGAGs found on membrane-associated heparan sulphate proteoglycans (HSPGs). Formation of a proper complex leads to activation of intracellular signalling pathways [5]. In this manner, HSGAGs, depending on their location and sequence context, can serve to either store FGF in an inactive form or promote FGF signalling through protein tyrosine kinase cell surface receptors.

All HSGAGs are characterized by repeating disaccharide building blocks, each of which consists of uronic acid attached to glucosamine by a 1 → 4 linkage. These carbohydrate polymers are structurally and chemically diverse, varying both in terms of the number of disaccharide units, as well as in the sulphation pattern present on each disaccharide. The glucosamine subcomponent of the disaccharide can be sulphated at the N, 3-O, and 6-O positions. Additionally, the uronic acid exists as one of two isomers (α -L-iduronic or β -D-glucuronic acid). The 2-O position of the uronic acid can also be either sulphated or unsulphated. These five potential binary modifications lead to an extraordinary level of sequence diversity in the nascent chain that together define the interactions between HSGAGs and various growth factors, morphogens and cytokines, including FGF2 [6]. As a result, HSGAG patterns and sequences have been implicated as important modulators in a wide range of normal and pathological biological processes [7,8]. Furthermore, HSGAG sequences expressed on the surface of cells and in the ECM are not static, but are rather dynamic, enabling a carefully controlled regulation of mitogenic signalling pathways [6].

Recent studies have elucidated some of the roles for HSGAGs in modulating FGF2-induced SMC and bovine aortic EC (BAEC) proliferation. Addition of exogenous highly sulphated HSGAGs, such as heparin [9], as well as lower sulphated HSGAGs derived from BAEC [10–12], such as perlecan, the major HSPG secreted by EC, have been found to inhibit FGF2-mediated

Abbreviations used: BAEC, bovine aortic endothelial cells; DMEM, Dulbecco's modified Eagle's medium; EC, endothelial cells; ECM, extracellular matrix; Erk, extracellular-signal-regulated kinase; FBS, fetal bovine serum; FGF, basic fibroblast growth factor; FGFR, FGF receptor; HSGAG, heparin/heparan sulphate-like glycosaminoglycan; HSPG, heparan sulphate proteoglycan; IL-3, interleukin-3; PI, proliferation index; RT, reverse transcriptase; SMC, smooth muscle cells.

¹ To whom correspondence should be addressed (e-mail rams@mit.edu).

proliferation of SMC [13]. The latter effect can be systematically mitigated via treatment of the conditioned media with specific HSGAG degrading enzymes [12,14]. Furthermore, HSGAGs at the SMC surface can serve dual functions, either inducing or inhibiting proliferation, depending on the biological context [15]. Exogenous heparin has also been reported to inhibit FGF2-mediated proliferation of EC [16]. Additionally, inhibition of HSGAG synthesis in BAEC abrogates the ability of FGF2 to induce a mitogenic response in this cell type [17]. Therefore, cell surface HSGAGs play an important role in regulating the responses of SMC and BAEC to FGF2 as well as mediating the interaction between the two cell types.

Previous work from our laboratory has demonstrated that SMC-derived HSGAGs modulate FGF2–HSGAG interactions through specific FGF receptor (FGFR) isoforms in a sequence-dependent manner [18]. The present study serves to extend these findings by determining how specific HSGAG structures on the cell surface of SMC, as opposed to those found on EC, influence FGF-induced proliferation. We found, in accordance with previous studies, that SMC-derived HSGAGs promote FGF-induced proliferation to a greater extent than EC-derived HSGAGs and that these activities are dependent on specific HSGAG structural motifs. We also found that these two cell types possess HSGAG structural differences that are substantially more pronounced within specific sequence contexts than in global composition. Furthermore, the ability of specific HSGAG fragments to support FGF2 signalling was found to be dependent on specific structural motifs released upon enzymic treatment. These findings provide insight into the ability for HSGAGs to greatly influence cell function through regulation of the activity of HSGAG-binding growth factors, such as FGF2. Finally, these experiments demonstrate that HSGAGs derived from distinct cell types, even those physiologically adjacent *in vivo*, elicit distinct proliferative responses.

EXPERIMENTAL

Proteins and reagents

Recombinant human FGF2 was a gift from Scios, Inc. (Mountain View, CA, U.S.A.). Recombinant heparinases were expressed as described previously [19]. Fetal bovine serum (FBS) was from Hyclone (Logan, UT, U.S.A.). L-Glutamine, penicillin, and streptomycin were obtained from GibcoBRL (Gaithersburg, MD, U.S.A.). Mouse recombinant interleukin-3 (IL-3) was from R & D Systems (Minneapolis, MN, U.S.A.). Dulbecco's modified Eagle's medium (DMEM), RPMI 1640, and PBS were from BioWhittaker (Walkersville, MD, U.S.A.). [³H]Thymidine and [¹²⁵I]Bolton-Hunter reagent were from New England Nuclear (Boston, MA, U.S.A.). Porcine intestinal mucosa heparin was purchased from Celsus Laboratories (Columbus, OH, U.S.A.). BaF3 cells transfected with FGFR1c were generously provided by Dr David Ornitz (Washington University, St. Louis, MO, U.S.A.). BAEC were generously provided by Dr Elazer Edelman (MIT, Cambridge, MA, U.S.A.).

Cell culture

Bovine aortic SMC were isolated from fresh calf aortas as described previously [20]. SMC were maintained in DMEM supplemented with 10 % (v/v) FBS, 100 µg/ml penicillin, 100 units/ml streptomycin and 500 µg/ml L-glutamine. SMC were grown in 75-cm² flasks at 37 °C in a 5 % CO₂ humidified incubator, and were passaged 2–3 times each week, when cells

reached confluence. SMC cultures were discarded after passage 8. BAEC were maintained in DMEM (low glucose), supplemented with penicillin (100 units/ml), streptomycin (100 µg/ml), glutamine (2 mM), and 10 % (v/v) FBS (HyClone), as described previously [13]. BAEC were used as long as they retained a cobblestone appearance at confluence [21] and elicited a robust response to FGF2.

BaF3 cells transfected with various FGFR isoforms were maintained as independent suspension cultures in propagation media composed of RPMI 1640 supplemented with 10 % (v/v) FBS, 100 µg/ml penicillin, 100 units/ml streptomycin, 500 µg/ml L-glutamine, and 500 ng mouse recombinant IL-3. Cultures were grown in 75-cm² flasks at 37 °C in a 5 % CO₂ humidified incubator and were passaged by dilution (1 : 10) three times each week.

Reverse transcriptase (RT)-PCR

Total RNA (5 µg) was isolated from SMC as well as from BAEC using Trizol reagent (Life Tech, Rockville, MD, U.S.A.) followed by reverse transcription with random hexamers. Specific oligomers were designed based on the published sequences of FGFR1c, FGFR2b, FGFR2c and FGFR3c (Genbank® accession numbers X51803, M97193, x52832 and M61881 respectively) in order to detect the expression of specific FGFR isoforms. Sequences of primer pairs corresponding to distinct FGFR isoforms were as follows: FGFR1c, 5'-TGGAGCTGGAAGTG-CCTCCTC-3' and 5'-GTGATGGGAGAGTCCGATAGA-3'; FGFR2b, 5'-GTCAGCTGGGGTCGTTTCATC-3' and 5'-CTGGTTGGCCTGCCCTATATA-3'; FGFR2c, 5'-GTCAGCTGGGGTCGTTTCATC-3' and 5'-GTGAAAGGATATCCCAAT-AGA-3'; FGFR3c, 5'-GTAGTCCCCGGCCTGCGTGCTA-3' and 5'-TCCTTGACAAATGTCACCTTT-3'. To control for total cell RNA, RT-PCR was also performed on β-actin using the primers 5'-GCCAGCTCACCATGGATGATGATAT-3' and 5'-GCTTGCTGATCCACATCTGCTGGAA-3'. PCR was performed using the Advantage-GC cDNA kit from Clontech according to the manufacturer's instructions (Palo Alto, CA, U.S.A.).

FGF2 binding assay

[¹²⁵I]Hunter-Bolton Reagent was used to radiolabel FGF2 as described previously [20]. A Sephadex G-10 column was used to separate labelled protein from unconjugated reagent. Protein fractions were pooled, the activity measured, and then the concentration was determined using an ELISA. Preparations were tested in a mitogenic assay to ensure biological activity and protein viability. Binding assays were performed as previously described for SMC [14], with an identical method used herein for BAEC. Briefly, SMC (or BAEC) were seeded in 24-well tissue culture plates and incubated for 72 h at 37 °C until near confluence. Cells were washed once in DMEM-supplemented medium lacking FBS, treated with 0.1–100 nM heparinase I, heparinase II or heparinase III in DMEM-supplemented medium with 0.1 % FBS, incubated for 30 min at 37 °C and then placed at 4 °C for 15 min. As described previously [19], experimental and control wells were treated with 2 ng and 2 µg of [¹²⁵I]FGF2 respectively. The cells were incubated for 2 h at 4 °C and subsequently washed three times with cold PBS (pH 7.4) to remove unbound ligand. The cells were then washed quickly with 0.5 ml of Hepes (pH 7.0, 2 M NaCl) per well to remove FGF2 bound to the low affinity receptors. Each well was washed twice with 0.5 ml of sodium acetate buffer (pH 4.0, 2 M NaCl) to remove FGF2 bound to high affinity receptors. Each wash was collected and counted on

a Wallace 1270 Gamma Counter. Procedures were repeated three times for each condition and each cell type.

Isolation of HSGAGs from SMC and BAEC

Cell surface HSGAG fragments were collected from SMC and BAEC using one of two methods. In the first procedure, fully confluent SMC were treated with 2 ml of trypsin/EDTA (GibcoBRL) per 75-cm² tissue culture flask for 2 min, collected with 13 ml of medium, centrifuged at 195 *g* for 3 min, and diluted (1 : 20) with supplemented DMEM-based medium after the supernatant was discarded. Aliquots of suspension culture (120 ml) were added to each of four 500-cm² tissue culture plates, which were incubated for 72 h at 37 °C in a 5 % CO₂ humidified incubator. Each plate was washed twice with 60 ml of PBS and subsequently treated with 12 ml of trypsin/EDTA. The plates were incubated at 37 °C for 25 min to harvest cell surface proteoglycans. The resulting cell/trypsin solution was collected and boiled for 10 min to deactivate the trypsin and other proteins in the solution. Subsequently, the solution was centrifuged at 4500 *g* for 5 min. The supernatant was collected and the pellet discarded. Samples were concentrated by centrifuging at 2800 *g* in a Centriprep 3 (Amicon, Beverly, MA, U.S.A.) for 6 h.

The purification of harvested proteoglycans was completed by anion-exchange chromatography. Briefly, 1 ml of DEAE-Sephacel (Pharmacia Biotech, Uppsala, Sweden) was added to a polypropylene pipette tip plugged with glass wool. The column was equilibrated with 5 column vol. of DEAE wash buffer (200 mM NaCl). The collected supernatant was added to the column. Non-specific binders were eluted with 20 column vol. of DEAE wash buffer. HSGAGs and associated proteoglycans were eluted with 5 column vol. of DEAE elution buffer (1 M NaCl). The collected samples were buffer-exchanged in distilled water using a Centriprep 3. Samples were stored at -20 °C until use.

To eliminate potentially confounding effects arising from the remnant proteoglycan protein core and other co-isolates, cell surface HSGAGs were isolated via a second procedure. Confluent SMC and BAEC were trypsinized and diluted (1 : 10) with supplemented DMEM medium into twelve 75-cm² tissue culture flasks per cell type. Cells were incubated until they reached full confluence, at which point each flask was washed twice with 20 ml of PBS, and the medium was replaced with 2 ml of PBS. Heparinase I, II or III was added to three flasks each for both SMC and BAEC, sufficient to yield final concentrations of 10 nM. An equivalent volume, 10 μ l, of PBS was added to the three remaining flasks for each cell type. SMC and BAEC were then incubated for 30 min and 9 h respectively, at 37 °C, sufficient time to yield the same concentration of HSGAG from each cell type. The preparations were collected and centrifuged at 4500 *g* for 10 min at 4 °C. The pellet was discarded, and the samples were boiled for 30 min to eliminate residual enzymic activity and inactivate proteins ordinarily in SMC or BAEC conditioned media. The HSGAG samples were filtered using a 0.45 μ m syringe filter and subsequently purified by anion-exchange chromatography as outlined above.

Quantification of isolated HSGAGs

Saccharide amounts of SMC- and BAEC-derived HSGAGs were quantified by Alcian Blue with slight modifications from a previously described method [22] and by the carbazole assay. Undiluted HSGAG samples, as well as those diluted at a ratio of 1 : 10, were used for the carbazole assay. An aliquot

(200 μ l) of each sample was supplemented with 20 μ l of 4 M ammonium sulphamate and 1 ml of 25 mM sodium tetraborate in H₂SO₄ in screw-cap vials. The solutions were boiled for 5 min and allowed to cool to room temperature. Carbazole [40 μ l of 7.5 % (w/v)] in 95 % ethanol was added to each solution. The mixtures were then boiled for 15 min and allowed to cool to room temperature. Absorbance was measured at 520 nm. Uronic acid concentrations were obtained by normalizing absorbance readings to a heparin standard curve produced during the analysis of cell surface HSGAG samples.

Compositional analysis

Cell surface HSGAG samples were treated with 200 nM heparinase I, II or III in a buffer composed of 25 mM sodium acetate, 100 mM NaCl and 5 mM calcium acetate, pH 7.0. Fragments were incubated with the various heparinases overnight at 30 °C to ensure complete digestion and analysed by capillary electrophoresis in reverse polarity using a running buffer composed of 50 mM Tris/phosphate and 10 μ M dextran sulphate, pH 2.5. Identities of the resultant saccharides were determined based on co-migration with known standards.

SMC and BAEC proliferation assays

Confluent SMC and BAEC were treated with 2 ml of trypsin/EDTA (GibcoBRL) per 75-cm² tissue culture flask for 2 min and centrifuged at 195 *g* for 3 min, after which the supernatant was discarded. Cells were resuspended in the appropriate proliferation medium, and plated at a 1 : 10 dilution, 1 ml/well in 24-well plates. The cells were then incubated for 24 h at 37 °C, 5 % CO₂, after which they were treated with 500 ng/ml HSGAG or 10 μ l of PBS and 5 ng/ml FGF2 or 10 μ l of PBS and incubated at 37 °C, 5 % CO₂ for 21 h. The concentrations added had been previously determined to induce a maximal response in these cell types. After the incubation, each well was treated with [³H]thymidine sufficient to yield a final concentration of 1 μ Ci. The cells were incubated for 3 h. Each well was washed three times with PBS and treated with 500 μ l of a 1 M NaOH solution until the cells lysed. The contents of each well were mixed with 5 ml of scintillation fluid and the mitogenic response was measured in counts per min as determined by a scintillation counter.

BaF3 cell proliferation assay with SMC and BAEC HSGAGs

BaF3 cells expressing FGFR1c were washed and resuspended to a density of 1 $\times$ 10⁵ cells/ml in supplemented DMEM. The cell suspension was divided into 24 samples, each of 6 ml. Cell samples were centrifuged at 195 *g* for 3 min at room temperature and resuspended in 3 ml of the cell-derived HSGAG preparations (from heparinase treatments) such that two sets of cells in each condition were produced, yielding a total of 6 distinct samples in duplicate for each cell type. One sample from each set of preparations was supplemented with 50 ng/ml FGF2 and the other was supplemented with an equivalent volume, 10 μ l, of PBS. A volume of 1 ml from each preparation was added to each of three wells on 24-well tissue culture plates. After incubating for 72 h at 37 °C, the whole cell number was determined by Coulter counter, and data from each of three experiments were normalized using a proliferation index (PI), as described previously [23]. The index is defined as the increase in cell number for the experimental case divided by the increase in cell number for the positive control. The proliferation index was calculated independently for each experiment.

Table 1 ^3H -incorporation into SMC and BAEC

SMC or BAEC were treated with 0–50 ng/ml FGF2, and proliferation was determined by thymidine incorporation. The effect of the various treatments was measured in counts per minute (c.p.m.). The percent increase is shown in parentheses.

	Thymidine incorporation (c.p.m.)	
	SMC	BAEC
– FGF	2780	14000
+ FGF (5 ng/ml)	11400 (310)	20700 (48)
+ FGF (50 ng/ml)	12400 (350)	21100 (51)

RESULTS

FGF2 induces a significant mitogenic response in both SMC and BAEC

Previous studies have identified that FGF2 is a potent mitogen for both SMC and EC. To confirm this observation and provide a basis for additional studies, we determined the proliferative effect of FGF2 on SMC and BAEC. Each cell type was incubated with 5 ng/ml of FGF2, and the extent of tritium incorporation over 3 h was measured. Consistent with previous reports [14,24], FGF2 promoted a significant mitogenic response in both SMC and BAEC (Table 1). While the background tritium incorporation for BAEC cells is higher, the relative increase in ^3H counts upon FGF2 stimulation is much greater (approx. 6-fold) for SMC than for BAEC. Dose–response studies, in which the amount of exogenous FGF2 added to the culture was varied by 10-fold, demonstrate that approximately the same amount of FGF2 is required to induce a maximal proliferative response in both SMC and BAEC. Thus, in terms of an FGF-inducible proliferative response, SMC appear more responsive to FGF2 stimulation than do BAEC as determined by tritium incorporation.

The above results were extended to include an analysis of intracellular signalling pathways induced by FGF2 stimulation of either SMC or BAEC. Both cell types were treated for 20 min with either FGF2 or PBS, and then activation of intracellular signalling pathways was assessed by Western blot. As shown in Figure 1, phospho extracellular-signal-regulated kinase 1 (Erk1) and Erk2, known markers of proliferation [25], were upregulated in both SMC and BAEC, but the effect was more pronounced in SMC, consistent with the more dramatic effect of FGF2 on SMC proliferation. Examination of other signalling pathways revealed similar findings (results not shown). Taken together, these results demonstrate that the administration of FGF2 induces a downstream activation of intracellular signalling pathways to an extent consistent with the observed proliferative response (i.e. tritium incorporation). These results point to the fact that while SMC respond more readily to exogenous FGF2 administration than do BAEC, the difference is in the extent of activation, rather than the kind of activation.

There are several possible explanations for this observed difference in the proliferative response of SMC versus BAEC. One possible explanation is that SMC and BAEC express distinct patterns of FGFR isoforms on their cell surface, and accordingly, the distinct receptor isoform repertoire modulates the extent of signalling induced by FGF2. Previous studies have identified that several FGFR isoforms, including FGFR1c, FGFR3c and FGFR4, efficiently mediate FGF2 binding to the cell surface and activation of intracellular signalling pathways leading to cell proliferation [26]. To test whether SMC and BAEC express different FGFRs, RT-PCR was performed on both SMC and BAEC to determine

Figure 1 Western blot of SMC and BAEC after FGF2 stimulation

SMC and BAEC were treated with 5 ng/ml FGF2 or an equivalent volume of PBS for 10 min, after which the cells were lysed, and a Western blot was performed utilizing antibodies specific to Erk1 and Erk2, known markers of proliferation.

Figure 2 RT-PCR of FGFRs expressed by SMC and BAEC

RT-PCR was performed using primers specific to four FGFR isoforms to determine the expression pattern of FGFRs by SMC and BAEC. Expression levels were controlled for total cell protein based on RT-PCR of β -actin. A, actin; 1C, 2B, 2C and 3C refer to the various FGFR isoforms.

their receptor expression profile. Both cell types were tested with specific probes to FGFR1c, FGFR2b, FGFR2c and FGFR3c. Since existing evidence indicates that FGFR4 is primarily present at sites other than the cell surface [27], it was not included in this study. As seen in Figure 2, both SMC and BAEC primarily express FGFR1c, with very little, if any, evidence of expression of other receptor isoforms. Therefore it seems unlikely that the observed differences in mitogenic response observed upon FGF2 stimulation of SMC and BAEC can be attributed to differences in isoforms of FGFR that are expressed at the cell surface of the two cell types.

Although the expression of FGFR isoforms is the same for SMC and BAEC, it is possible that SMC and EC express different quantities of FGFR1c on their cell surfaces. However, the signal intensity derived from RT-PCR of FGFR1c is similar in EC and SMC, especially when referenced to an actin control. To address this issue further and confirm this conclusion, FGF2 binding to low- and high-affinity receptors on SMC and BAEC was determined using an [^{125}I]FGF2 binding assay. We find that FGF2 binding to both high- and low-affinity receptors is similar for both SMC and BAEC (Figure 3). Given that these cells primarily express only one receptor isoform, the similarity in binding affinity suggests that both cell types display a similar quantity of receptors on their surface. Taken together, these results point to the fact that the difference in the proliferative response between SMC and EC does not seem to be primarily related to differential FGFR expression. As such, we sought to determine whether differences in cell surface HSGAGs, important for formation of an active signalling complex, could account for the marked differences in FGF-mediated signalling between SMC and BAEC.

Cell surface HSGAGs can be efficiently harvested

HSGAGs were harvested from SMC and BAEC by two methods. The first method employed trypsin digestion of the cell surface

Figure 3 FGF2 binding to SMC and BAEC-expressed FGFR

SMC (black bars) and BAEC (grey bars) were incubated with 2 ng [125 I] FGF2 for 2 h at 4 °C. Bound [125 I] FGF2 was released from separately from low affinity (LA) receptors and high affinity (HA) receptors. The amount of ligand bound was determined at 0 h and 5 h after incubation with [125 I] FGF2. CPM, counts per minute.

to cleave the proteoglycan core of membrane-bound HSGAGs, liberating intact HSGAG chains coupled to parts of the proteoglycan protein core. After boiling, to inactivate the trypsin and other potential contaminating proteins, the suspensions were concentrated using a 3000 Da molecular mass cut-off filter. The solutions were then purified by anion-exchange chromatography and reconcentrated. Yields were determined by Alcian Blue and carbazole assays as described in the Experimental section. Given the nature of this isolation procedure, other polysaccharides, such as chondroitin sulphate and dermatan sulphate, could be isolated, especially considering that perlecan, found prominently on vascular basement membranes, contains both heparan sulphate as well as chondroitin sulphate [28]. In order to confirm and extend our results using trypsin-derived material, a second harvesting method was employed wherein SMC and BAEC were treated with heparinase I, II, III or PBS. Since heparinases I–III have unique substrate specificity and are anticipated to release structurally distinct HSGAG fragments, this methodology also provides some information about the influence of HSGAG fine structure on FGF-mediated proliferation of BAEC and SMC. With each enzyme application, treatment times were set to liberate an identical amount of HSGAGs from each cell type. Cell-free HSGAG solutions were collected and boiled to eliminate residual enzyme activity as well as the activity of any proteins potentially secreted during enzymic treatment. Subsequently, the solutions were filtered through 0.45 μ m syringe filters. The method employed preferentially releases HSGAGs over other glycosaminoglycan polysaccharides because the activities of heparinases are specific to this type of glycosaminoglycan [29]. Solutions containing heparinase-derived HSGAG fragments were purified and concentrated by anion-exchange chromatography followed by dialysis in a similar manner to the trypsin-derived HSGAG material.

To confirm the absence of contaminating activities in the various HSGAG preparations that might compromise further analysis, all material was tested for its mitogenic potential in the absence of exogenous ligand using a BaF3 cell line. Based on previous observations [18], we anticipated that HSGAG material of sufficient purity would not induce a significant proliferative (or inhibitory) response. BaF3 cells transfected with FGFR1c were washed three times in media lacking IL-3 and plated at a density of 1×10^5 cells/ml. Cells were then treated with either porcine mucosa heparin or cell-derived HSGAGs at a final concentration of 500 ng/ml. After incubating for 72 h,

proliferation was measured by whole cell counts, as determined by an electronic cell counter. We found that none of the cell surface HSGAG fractions significantly induced a proliferative or inhibitory response (results not shown). These findings suggest that the cell-derived HSGAG fractions do not produce a proliferative response in the absence of exogenous ligand, and are therefore sufficiently free of contaminating activities that would potentially complicate analysis.

SMC proteoglycans promote FGF-induced signalling

To test further whether observed differences in FGF-mediated cellular proliferation between SMC and BAEC could be due, at least in part, to differences in their respective HSGAG composition [30], the effect of trypsin-harvested SMC and BAEC HSGAG on cell mitogenesis was examined. In this case, recipient SMC or BAEC were first treated with chlorate to suppress endogenous HSGAG production. Then, exogenous FGF2, in combination with either SMC- or BAEC-derived HSGAGs, was added to chlorate-treated cells. In a prior experiment, SMC and BAEC were treated with varying amounts of chlorate to determine the chlorate level that was effective at abrogating HSGAG production without being cytotoxic. We found that cellular responses to the addition of exogenous FGF2, as well as sulphation of HSGAGs at the cell surface, were maximally reduced in media supplemented with 50 mM sodium chlorate. Furthermore, both SMC and BAEC that were chlorate treated possessed a similar cell morphology to untreated controls and maintained a parallel proliferative capacity in the absence of exogenously-added ligand. Together, these results suggest that 50 mM chlorate has very few, if any, non-specific cytotoxic effects on the cells. Notably, however, in both cell types, tritium incorporation upon addition of 5 ng/ml FGF2 was dramatically suppressed, such that it was statistically indistinguishable from negative controls, indicating the effective suppression of functional HSGAGs on the cell surface.

Having confirmed that chlorate-treated SMC and BAEC lack fully-sulphated (and functional) HSGAGs at their cell surface, we examined the effect of the addition of trypsin-derived SMC or BAEC HSGAGs on FGF-induced proliferation. To test this, chlorate-treated SMC and BAEC were supplemented with FGF2, as well as with isolated HSGAGs from either SMC or BAEC. We found that when chlorate-treated SMC cells were treated with HSGAGs derived from SMC in addition to FGF2, a significant increase in tritium incorporation was observed above the negative control (Figure 4). In fact, the observed increase rivalled the response observed when FGF and exogenous heparin were added to chlorate-treated SMC. Conversely, when chlorate-treated BAEC cells were treated with exogenous BAEC HSGAGs and FGF2, no significant tritium incorporation was observed above the negative control. Importantly, SMC HSGAGs added to chlorate-treated BAEC yielded a response that was significantly above the negative control, indicating that SMC proteoglycans can support FGF2-induced proliferation even when added to BAEC. Together, these results suggest that HSGAG proteoglycans from the cell surface of SMC cells facilitate FGF2-mediated signalling to a greater extent than BAEC-derived HSGAGs. We examined whether the observed functional differences could be correlated with differences in HSGAG fine structure.

Determination of HSGAG proteoglycan fine structure that promotes FGF2 signalling

The composition of the cell surface HSGAGs from SMC and BAEC was assessed using enzymic treatment and capillary

Figure 4 Effect of cell-derived HSGAG on FGF2-mediated SMC and BAEC mitogenicity

SMC and BAEC were incubated with 50 mM sodium chlorate to inhibit endogenous HSGAG production. Cells were supplemented with (from left to right) PBS, 5 ng/ml FGF2 and 500 ng/ml heparin, 5 ng/ml FGF2 and 500 ng/ml SMC-derived HSGAG, or 5 ng/ml FGF2 and 500 ng/ml BAEC-derived HSGAG. The mitogenic response was determined by [3 H]thymidine incorporation. CPM, counts per minute.

Figure 5 Compositional analysis of SMC (upper) and BAEC (lower) as determined by exhaustive digestion of the sample with a heparinase cocktail and separation of the resulting disaccharides by capillary electrophoresis

The observed disaccharides were identified by co-migration with known standards: (1) $\Delta U_{25}H_{NS,6S}$; (2) $\Delta U_{25}H_{NS}$; (3) $\Delta UH_{NS,6S}$; (4) ΔUH_{NS} ; (5) $\Delta UH_{NAC,6S}$.

electrophoresis according to a previously defined methodology [31]. We found that the cell surface HSGAGs of SMC and BAEC, isolated by trypsin treatment, although similar in structure, display subtle differences in the relative amounts of disaccharide building blocks (Figure 5). For example, though the HSGAGs of BAEC

Table 2 Compositional analysis of HSGAG fragments from SMC or BAEC after heparinase I, II or III treatment

The composition of the various pools was determined by capillary electrophoresis after full digestion by heparinase I, II and III (hepl, II and III respectively).

Disaccharide	Composition (% of total)					
	SMC			EC		
	HepI	HepII	HepIII	HepI	HepII	HepIII
$\Delta U_{25}H_{NS,6S}$	5	4	16	2	3	8
$\Delta U_{25}H_{NS}$	22	24	30	22	34	33
$\Delta UH_{NS,6S}$	6	8	8	6	3	5
$\Delta U_{25}H_{NAC,6S}$	0	0	0	0	0	0
ΔUH_{NS}	31	18	20	33	29	30
$\Delta U_{25}H_{NAC}$	0	0	0	0	0	0
$\Delta UH_{NAC,6S}$	8	12	7	6	4	2
ΔUH_{NAC}	28	34	19	31	27	22

and SMC both contain the trisulphated disaccharide $\Delta U_{25}H_{NS,6S}$, it is present in higher amounts in SMC HSGAGs. Similar trends are observed for several of the other disaccharide constituents, namely $\Delta UH_{NS,6S}$ and $\Delta UH_{NAC,6S}$. Taken together, these results suggest that functional differences in SMC versus BAEC HSGAGs can, at least partially, stem from the fine structure of the HSGAGs at their respective cell surface.

To extend the above findings to include an examination of the fine structure of HSGAGs at the cell surface of BAEC and SMC, we determined whether fragments of specific structure, released from the cell surface, would promote or inhibit FGF2-induced proliferation. To this end, we utilized the heparinases from *Flavobacterium heparinum*, a family of three enzymes that depolymerize HSGAGs with defined substrate specificities. Heparinase I cleaves highly-sulphated, or heparin-like, domains leaving intact undersulphated, or heparan-like, regions. Conversely, heparinase III digests regions of low sulphation, and heparinase II cleaves both heparin- and heparan-like domains [29]. SMC treated with heparinase III prior to introduction of FGF incorporated significantly more tritium than those not treated with heparinase III prior to addition of FGF (results not shown). A similar effect was not observed with heparinase I treatment, suggesting that the observed effect was due to the unique substrate specificity of heparinase III. Indeed, compositional analysis of heparinase I-, II- and III-released fragments indicated that heparinase III-derived fragments were higher in 2-O sulphation than those derived from heparinase II and especially heparinase I (Table 2), consistent with the published substrate specificity of the heparinases. Previous studies have implicated 2-O sulphation in high affinity FGF binding [32,33]. Notably, SMC HSGAGs had a higher degree of 6-O sulphation when isolated by heparinase II or III treatment than those isolated using heparinase I treatment. This sulphate group has also been associated with FGF2 activity through FGFR1 [34].

Further confirmation that cell surface HSGAGs from SMC and BAEC are distinct arises from the fact that heparinase III treatment of BAEC cannot recapitulate the effect observed with SMC HSGAGs, namely the promotion of tritium incorporation by heparinase III treatment of cell surface HSGAG. Compositional analysis of BAEC HSGAG fragments released after heparinase I, II or III treatment, wherein differential cleavage of HSGAGs enables the examination of the fine structure of the building block sequences, confirmed that these fragments are structurally distinct from SMC-derived HSGAG oligosaccharides (Table 2).

Figure 6 FGF2-mediated proliferation of BaF3 cells expressing FGFR1c

Transfected BaF3 cells were incubated in the presence of 50 ng/ml FGF-2 and cell derived HSGAG fragments from SMC (black bars) or BAEC (grey bars). Fragments were isolated by treatment with 10 mM heparinase I (HepI), II (HepII) or III (HepIII), or an equivalent volume of PBS. Proliferation is represented by PI, as described in the Experimental section. * $P < 0.05$.

BaF3 cells provide an appropriate model to analyse the effect of cell derived HSGAGs on FGF2-mediated mitogenic activity via different FGFRs

The ability of heparinase treatment to markedly influence cell proliferation differently in SMC and BAEC suggested that the fine structure of the cell surface HSGAGs may influence FGF2-mediated responses. To examine this possibility we employed a simplified system to more accurately assess the ability of subsets of the HSGAG pool to induce an FGF2 response. To this end, activity was tested with proliferation assays using BaF3 cells engineered to express FGFR1c [26]. The use of BaF3 cells enabled the introduction of a simple, clean, and biologically relevant system, as these cells lack cell surface HSGAGs and express only the transfected FGFR. The proliferative potential of these cells was characterized by culturing them in the presence or absence of FGF2, with or without HSGAGs. In this case, porcine intestinal mucosa heparin was used as a positive control [23].

The biological activity of the cell derived HSGAGs was measured by whole-cell proliferation over a 3-day period when transfected BaF3 cells were cultured with cell surface HSGAGs and FGF2. The cell counts were converted to a PI. All values are expressed as PI, where a value of 1 indicates the 'maximal' proliferative response, as induced by FGF2 and heparin, and a PI of 0 indicates a negligible response, equivalent to that of untreated cells. The formula for the PI has been described previously [23]. The reference point of $PI = 1$ was from BaF3 cells stimulated with 50 ng/ml FGF2 and 500 ng/ml heparin. This reference point allows for the proliferative response to be standardized, enabling comparisons between HSGAG preparations.

SMC HSGAG fragments derived by treatment with either heparinase II or III consistently elicited a significantly higher PI than the PBS control in BaF3 cells transfected with FGFR1c in the presence of FGF2 (Figure 6). The proliferative response induced by these fragments was similar to that induced by exogenous heparin. Heparinase I-derived HSGAG fragments, however, did not yield a response greater than that of the PBS control. Conversely, BAEC HSGAGs derived by heparinase III treatment (as well as HSGAGs obtained from heparinase I treatment) did not elicit a response significantly greater than that of the PBS control, confirming our findings that highly sulphated HSGAGs

from the cell surface of SMC were most efficient at promoting FGF2-induced cellular proliferation. The reproducibility of this *in vitro* data suggests that similar HSGAG fractions are obtained with different isolations.

DISCUSSION

The HSGAGs in the ECM and at the cell surface have been shown to act as co-receptors in FGF signalling by binding to both FGF and FGFR [35–37] and forming a ternary complex at the cell surface. Through the use of cells deficient in aspects of HSGAG biosynthesis, it has been demonstrated that growth factors, including FGF2, depend on cell surface HSGAGs for their mitogenic activity [5,38,39]. Furthermore, HSGAGs serving the co-receptor function have been suggested to influence the strength and type of cellular response induced by FGF. The ability of the HSGAG co-receptor to mediate FGF binding and activity is dependent on both the location and composition of the HSGAG in question. Not only do cell surface and soluble, ECM-derived HSGAGs appear to function differently [15], but studies have demonstrated that subtle modifications in HSGAG structure can alter their ability to induce a cellular response [40,41]. Therefore alterations in composition and sequence within a given HSGAG subset (ECM as opposed to cell surface) may alter the capacity of the HSGAG to alter function as appropriate to its location.

Using a variety of cell types as well as *in vitro* binding assays, previous studies have addressed the structural requirements for HSGAG binding to FGF2. In general, these studies have found that HSGAG–FGF2 interactions are thought to be determined by the N- and 2-O-sulphate content of the saccharide chain [32,33]. In addition, others have indicated that activation of FGFR1 by FGF2 can be prevented by selective elimination of 6-O sulphate groups [34]. These studies have determined the structural requirements for high affinity FGF binding and provided some context to fragments that promote FGF activity. However, many of these studies utilized fully sulphated heparin as a surrogate for cell surface heparan sulphate, or they added exogenous heparan sulphate to cells that already expressed a repertoire of HSGAGs at their cell surface, thus failing to address FGF activity within the context of these cell surface HSGAGs. Significantly, the membrane association of HSPGs has been suggested to determine the capacity of a given HSGAG to promote an FGF2 response [42]. The previously reported inhibitory effects of FGF2 are associated with soluble HSPGs, including constitutively shed syndecans and glypicans [43,44], whereas HSPGs that promote the response, even with the same protein core, are typically cell surface in nature [45,46]. As such, these studies provided an important foundation for additional work that probes the fine structure of HSGAGs at the cell surface. In this study, we sought to extend previous results by directly probing whether HSGAG fine structure at the cell surface modulates FGF-mediated cell proliferation and whether the structural rules developed using exogenous heparin fragments are consistent with reference to HSGAGs at the cell surface.

Thus there are three major findings of this paper. First, we find that HSGAGs at the surface of SMC and BAEC are one of the primary regulators of FGF-mediated signalling and, as such, they regulate the 'output' of FGF binding to its cell surface receptor. Second, we find that this differential regulation is due to differences in the fine structure of HSGAGs. We find that the global composition of BAEC and SMC is fairly similar, yet we show here that HSGAGs isolated from each have a profoundly different effect on FGF-mediated proliferation. Using the heparinases, we have mapped the fine structure of both and confirm the suggestion that it is not simply composition, but

also structural elements, that dictate the ability of HSGAG to promote FGF signalling. Finally, this study represents a confirmation and extension of several previous findings that the ability of a HSGAG fragment to mediate FGF binding and activity is correlated with 2-O- and, to a lesser extent, 6-O-sulphation of the polysaccharide chain. Given that structural studies have shown that promitogenic sequences of HSGAGs bind to both FGF2 and FGFR1 at distinct sites on the carbohydrate chain, the possibility existed that HSGAGs with similar or even identical compositions can, through the presence or absence of distinct sequences, have different capacities to induce a cellular response. Using SMC and BAEC, we confirm that this is possible in that the global composition of the HSGAGs of these cell types is similar, yet, due to differences in fine structure, they possess a markedly different ability to promote FGF-induced proliferation.

In the context of the structural formation of a ternary complex, the differential effects exhibited between the cell types, given the gross similarities in HSGAG composition but differences in fine structure, suggest that the sequence of BAEC HSGAGs is one in which disaccharides with sulphate groups necessary for ligand binding and those required for receptor activation are sufficiently far apart to prevent activation while enabling binding. These results therefore imply that either ligand-receptor interactions are highly sensitive to specific compositional patterns, or specific HSGAG sequences are required to optimally bind FGF2 and/or induce a response through FGFR1c [47], supporting the notion that a proper spatial display of sulphated groups promotes optimal ligand-receptor interactions [18].

As shown in this study, the ability of bovine aortic SMC and BAEC to respond differently to FGF2, as mediated by their cell surface HSGAGs, has potential implications in pathological settings. In the normal aorta, EC and SMC share the same physiological microenvironment. Extrapolating the results of this study suggests that theoretically, in a setting conducive to hyperplasia, the enhanced response to FGF2 imparted by SMC HSGAGs, as opposed to those from BAEC, suggests that SMC would undergo proliferation much more readily. Under normal conditions, EC HSPGs are necessary for the inhibition of SMC [48]. Importantly, this supports the notion that the differential responses of SMC and EC *in vivo* could be a function of their distinct HSGAG compositions. Given the capacity for variable HSGAG compositions to affect both FGF2-FGFR1 binding, and therefore the ability of FGF2 to induce a biological response, a further understanding of the specific nature of HSGAG fragments defining given levels of response will provide further insight into the nature of the induction of FGF2 signalling through the ternary complex, as well as the pathogenesis of proliferative vascular disorders such as atherosclerosis.

This work was supported in part by funding from NIH grants HL59966 and CA 090940 (to R.S.), the Burroughs Wellcome Foundation (to R.S.), Howard Hughes Medical Institute Pre-doctoral Fellowship (D.B.), and Merck/MIT fellowship (to Z.S.).

REFERENCES

- Ross, R. (1993) The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* (London) **362**, 801–809.
- Conrad, H. E. (1998) *Heparin-Binding Proteins*, Academic Press, San Diego.
- Saksela, O., Moscatelli, D., Sommer, A. and Rifkin, D. B. (1988) Endothelial cell-derived heparan sulfate binds basic fibroblast growth factor and protects it from proteolytic degradation. *J. Cell Biol.* **107**, 743–751.
- Gimbrone, M. A. (1995) Vascular endothelium in health and disease. In *Molecular Cardiovascular Medicine* (Haber, E., ed.), pp. 49–61, Scientific American Medicine, New York.
- Yayon, A., Klagsbrun, M., Esko, J. D., Leder, P. and Ornitz, D. M. (1991) Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. *Cell* **64**, 841–848.
- Perrimon, N. and Bernfield, M. (2000) Specificities of heparan sulphate proteoglycans in developmental processes *Nature* (London) **404**, 725–728.
- Hahnenberger, R., Jakobson, A. M., Ansari, A., Wehler, T., Svahn, C. M. and Lindahl, U. (1993) Low-sulphated oligosaccharides derived from heparan sulphate inhibit normal angiogenesis. *Glycobiology* **3**, 567–573.
- Lin, X., Buff, E. M., Perrimon, N. and Michelson, A. M. (1999) Heparan sulfate proteoglycans are essential for FGF receptor signaling during *Drosophila* embryonic development. *Development* **126**, 3715–3723.
- Majack, R. A. and Clowes, A. W. (1984) Inhibition of vascular smooth muscle cell migration by heparin-like glycosaminoglycans. *J. Cell. Physiol.* **188**, 253–256.
- Edelman, E. R., Adams, D. H. and Karnovsky, M. J. (1990) Effect of controlled adventitial heparin delivery on smooth muscle cell proliferation following endothelial injury. *Proc. Natl. Acad. Sci. U.S.A.* **87**, 3773–3777.
- Forsten, K. E., Courant, N. A. and Nugent, M. A. (1997) Endothelial proteoglycans inhibit bFGF binding and mitogenesis. *J. Cell. Physiol.* **172**, 209–220.
- Han, R. O., Ettenson, D. S., Koo, E. W. and Edelman, E. R. (1997) Heparin/heparan sulfate chelation inhibits control of vascular repair by tissue-engineering endothelial cells. *Am. J. Physiol.* **273**, H2586–H2595.
- Nugent, M. A., Nugent, H. M., Iozzo, R. V., Sanchack, K. and Edelman, E. R. (2000) Perlecan is required to inhibit thrombosis after deep vascular injury and contributes to endothelial cell-mediated inhibition of intimal hyperplasia. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 6722–6727.
- Nugent, M. A., Karnovsky, M. J. and Edelman, E. R. (1993) Vascular cell-derived heparan sulfate shows coupled inhibition of basic fibroblast growth factor binding and mitogenesis in vascular smooth muscle cells. *Circ. Res.* **73**, 1051–1060.
- Fannon, M., Forsten, K. E. and Nugent, M. A. (2000) Potentiation and inhibition of bFGF binding to heparin: a model for regulation of cellular response. *Biochemistry* **39**, 1434–1445.
- Giroux, J. L., Matou, S., Bros, A., Tapon-Bretaudiere, J., Letourneur, D. and Fischer, A. M. (1998) Modulation of human endothelial cell proliferation and migration by fucoidan and heparin. *Eur. J. Cell Biol.* **77**, 352–359.
- Fujiwara, Y. and Kaji, T. (1999) Possible mechanism for lead inhibition of vascular endothelial cell proliferation: a lower response to basic fibroblast growth factor through inhibition of heparan sulfate synthesis. *Toxicology* **133**, 147–157.
- Berry, D., Kwan, C. P., Shriver, Z., Venkataraman, G. and Sasisekharan, R. (2001) Distinct heparan sulfate glycosaminoglycans are responsible for mediating fibroblast growth factor-2 biological activity through different fibroblast growth factor receptors. *FASEB J.* **15**, 1422–1424.
- Natke, B., Venkataraman, G., Nugent, M. A. and Sasisekharan, R. (2000) Heparinase treatment of bovine smooth muscle cells inhibits fibroblast growth factor-2 binding to fibroblast growth factor receptor but not FGF-2 mediated cellular proliferation. *Angiogenesis* **3**, 249–257.
- Nugent, M. A. and Edelman, E. R. (1992) Kinetics of basic fibroblast growth factor binding to its receptor and heparan sulfate proteoglycan: a mechanism for cooperativity. *Biochemistry* **31**, 8876–8883.
- Ashikaga, T., Strada, S. J. and Thompson, W. J. (1997) Altered expression of cyclic nucleotide phosphodiesterase isozymes during culture of aortic endothelial cells. *Biochem. Pharmacol.* **54**, 1071–1079.
- Bjornsson, S. (1998) Quantitation of proteoglycans as glycosaminoglycans in biological fluids using an Alcan blue dot analysis. *Anal. Biochem.* **256**, 229–237.
- Padera, R., Venkataraman, G., Berry, D., Godvarti, R. and Sasisekharan, R. (1999) FGF-2/fibroblast growth factor receptor/heparin-like glycosaminoglycan interactions: a compensation model for FGF-2 signaling. *FASEB J.* **13**, 1677–1687.
- Xin, L., Xu, R., Zhang, Q., Li, T. P. and Gan, R. B. (2000) Kringle 1 of human hepatocyte growth factor inhibits bovine aortic endothelial cell proliferation stimulated by basic fibroblast growth factor and causes cell apoptosis. *Biochem. Biophys. Res. Commun.* **277**, 186–190.
- Lin, L. L., Wartmann, M., Lin, A. Y., Knopf, J. L., Seth, A. and Davis, R. J. (1993) cPLA2 is phosphorylated and activated by MAP kinase. *Cell* **72**, 269–278.
- Ornitz, D. M., Xu, J., Colvin, J. S., McEwen, D. G., MacArthur, C. A., Coulier, F., Gao, G. and Goldfarb, M. (1996) Receptor specificity of the fibroblast growth factor family. *J. Biol. Chem.* **271**, 15292–15297.
- Citores, L., Wesche, J., Kolpakova, E. and Olsnes, S. (1999) Uptake and intracellular transport of acidic fibroblast growth factor: evidence for free and cytoskeleton-anchored fibroblast growth factor receptors. *Mol. Biol. Cell* **10**, 3835–3848.
- Iozzo, R. V. (1998) Matrix proteoglycans: from molecular design to cellular function. *Annu. Rev. Biochem.* **67**, 609–652.

- 29 Ernst, S., Langer, R., Cooney, C. L. and Sasisekharan, R. (1995) Enzymatic degradation of glycosaminoglycans. *Crit. Rev. Biochem. Mol. Biol.* **30**, 387–444
- 30 Chang, Z., Meyer, K., Rapraeger, A. C. and Friedl, A. (2000) Differential ability of heparan sulfate proteoglycans to assemble the fibroblast growth factor receptor complex in situ. *FASEB J.* **14**, 137–144
- 31 Keiser, N., Venkataraman, G., Shriver, Z. and Sasisekharan, R. (2001) Direct isolation and sequencing of specific protein-binding glycosaminoglycans. *Nat. Med.* **7**, 123–128
- 32 Turnbull, J. E., Fernig, D. G., Ke, Y., Wilkinson, M. C. and Gallagher, J. T. (1992) Identification of the basic fibroblast growth factor binding sequence in fibroblast heparan sulfate. *J. Biol. Chem.* **267**, 10337–10341
- 33 Wang, H., Toida, T., Kim, Y. S., Capila, I., Hilemna, R. E., Berfield, M. and Lindhart, R. J. (1997) Glycosaminoglycans can induce fibroblast growth factor-2 mitogenicity without significant growth factor binding. *Biochem. Biophys. Res. Commun.* **235**, 369–373
- 34 Lundin, L., Larsson, H., Kreuger, J., Kanda, Lindahl, U., Salmivirta, M. and Claesson-Welsh, L. (2000) Selectively desulfated heparin inhibits fibroblast growth factor-induced mitogenicity and angiogenesis. *J. Biol. Chem.* **275**, 24653–24660
- 35 Faham, S., Hileman, R. E., Fromm, J. R., Lindhart, R. J. and Rees, D. C. (1996) Heparin structure and interactions with basic fibroblast growth factor. *Science (Washington D.C.)* **271**, 1116–1120
- 36 Ornitz, D. M., Herr, A. B., Nilsson, M., Westman, J., Scahn, C. M. and Waksman, G. (1995) FGF binding and FGF receptor activation by synthetic heparan-derived di- and trisaccharides. *Science (Washington D.C.)* **268**, 432–436
- 37 Kan, M., Wang, F., Xu, J., Crabb, J. W., Hou, J. and McKeenan, W. L. (1993) An essential heparin-binding domain in the fibroblast growth factor receptor kinase. *Science (Washington D.C.)* **259**, 1918–1921
- 38 Rapraeger, A. C., Kruffka, A. and Olwin, B. B. (1991) Requirement of heparin sulfate for bFGF-mediated fibroblast growth and myoblast differentiation. *Science (Washington D.C.)* **252**, 1705–1708
- 39 Ishihara, M. (1994) Structural requirements in heparin for binding and activation of FGF-1 and FGF-4 are different from that for FGF-2. *Glycobiology* **4**, 817–824
- 40 Nurcombe, V., Smart, C. E., Chipperfield, H., Cool, S. M., Boilly, B. and Hondermarck, H. (2000) The proliferative and migratory activities of breast cancer cells can be differentially regulated by heparan sulfates. *J. Biol. Chem.* **275**, 30009–30018
- 41 Pye, D. A., Vives, R. R., Turnbull, J. E., Hyde, P. and Gallagher, J. T. (1998) Heparan sulfate oligosaccharides require 6-O-sulfation for promotion of basic fibroblast growth factor mitogenic activity. *J. Biol. Chem.* **273**, 22936–22942
- 42 Zhang, Z., Coomans, C. and David, G. (2001) Membrane heparan sulfate proteoglycan-supported FGF2-FGFR1 signaling: evidence in support of the 'cooperative end structures' model. *J. Biol. Chem.* **276**, 41921–41929
- 43 Mertens, G., Van der Schueren, B., van den Berghe, H. and David, G. (1996) Heparan sulfate expression in polarized epithelial cells: the apical sorting of glypican (GPI-anchored proteoglycan) is inversely related to its heparan sulfate content. *J. Cell Biol.* **132**, 487–497
- 44 Fitzgerald, M. L., Wang, Z., Park, P. W., Murphy, G. and Bernfield, M. (2000) Shedding of syndecan-1 and -4 ectodomains is regulated by multiple signaling pathways and mediated by a TIMP-3-sensitive metalloproteinase. *J. Cell Biol.* **148**, 811–824
- 45 Clasper, S., Vekemans, S., Fiore, M., Plebanski, M., Wordsworth, P., David, G. and Jackson, D. G. (1999) Inducible expression of the cell surface heparan sulfate proteoglycan syndecan-2 (fibroglycan) on human activated macrophages can regulate fibroblast growth factor action. *J. Biol. Chem.* **274**, 24113–24123
- 46 Bonne-Barkay, D., Shlissel, M., Berman, B., Shaoul, E., Admon, A., Vlodavsky, I., Carey, D. J., Asundi, V. K., Reich-Slotky, R. and Ron, D. (1997) Identification of glypican as a dual modulator of the biological activity of fibroblast growth factors. *J. Biol. Chem.* **272**, 12415–12421
- 47 Ostrovsky, O., Berman, B., Gallagher, J., Mulloy, B., Fernig, D. G., Delehedde, M. and Ron, D. (2001) Differential effects of heparin saccharides on the formation of specific fibroblast growth factor (FGF) and FGF receptor complexes. *J. Biol. Chem.* **277**, 2444–2453
- 48 Ettenson, D. S., Koo, E. W. Y., Januzzi, J. L. and Edelman, E. R. (2000) Endothelial heparan sulfate is necessary but not sufficient for control of vascular smooth muscle cell growth. *J. Cell. Physiol.* **184**, 93–100

Received 11 November 2002/11 March 2003; accepted 27 March 2003

Published as BJ Immediate Publication 27 March 2003, DOI 10.1042/BJ20021760