Fibroblast growth factor-2 binds to small heparin-derived oligosaccharides and stimulates a sustained phosphorylation of p42/44 mitogen-activated protein kinase and proliferation of rat mammary fibroblasts

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We examine the relationship between the chain length of heparin-derived oligosaccharides, fibroblast growth factor (FGF)-2 binding kinetics and the ability of the oligosaccharides to allow FGF-2-induced proliferation of chlorate-treated rat mammary fibroblasts. First, using an optical biosensor, we show that FGF-2 did not bind disaccharides, but definitively bound to tetrasaccharides. As the chain length increased from tetrasaccharide to octasaccharide, there was a substantial increase in k_{ass} ($564\,000\text{ M}^{-1}\cdot\text{s}^{-1}$ to $2\,000\,000\text{ M}^{-1}\cdot\text{s}^{-1}$, respectively) and affinity (K_d 77 nM to 11 nM, respectively) for FGF-2. From decasaccharides and longer, the k_{ass} and affinity for FGF-2 was reduced substantially (tetradecasaccharide k_{ass} $470\,000\text{ M}^{-1}\cdot\text{s}^{-1}$, K_d 30 nM). In chlorate-treated, and hence sulphated, glycosaminoglycan-deficient cells, FGF-2 alone or in the presence of disaccharides did not stimulate DNA synthesis and it only elicited an early transient dual phosphorylation of p42/44 mitogen-activated

protein kinase (MAPK). In the same cells FGF-2 in the presence of tetrasaccharides and longer oligosaccharides was able to restore DNA synthesis and enable the sustained dual phosphorylation of p42/44^{MAPK}. However, the oligosaccharides from tetrasaccharides to octasaccharides were less potent in proliferation assays than deca- and longer oligosaccharides. Therefore, there was no correlation between the binding parameters and the potency of the oligosaccharides in DNA synthesis assays. These results demonstrate that tetrasaccharides are able to bind FGF-2 and enable FGF-2 to stimulate cell proliferation, which sets important boundary conditions for models of the FGF-2–heparan sulphate–FGF receptor complex.

Key words: binding kinetics, fibroblast growth factor receptor, glycosaminoglycan, heparan sulphate, optical biosensor.

INTRODUCTION

The fibroblast growth factors (FGFs) regulate cell fate during a large number of critical steps in development. The importance of these growth factors is underlined by the fact that ectopic signalling by FGFs and FGF receptors (FGFRs) results in pathologies as diverse as skeletal growth defects and carcinomas [1]. FGF-2 possesses a dual receptor system consisting of tyrosine kinase receptors (FGFR) and heparan sulphate (HS) proteoglycans and it is generally accepted that both receptors must be present for FGF-2 to trigger a proliferative response [1,2]. However, the molecular mechanism that underpins this latter requirement remains controversial. In broad terms, three distinct models are proposed to explain how FGF and HS may co-operate to induce the dimerization of the FGFR and thus activate its intracellular kinase. In the ‘growth hormone’ model, a single FGF dimerizes two FGFRs, with HS binding both growth factor and receptor [3,4]. In the ‘HS-dependent dimerization’ model, the HS chain binds two FGFs and the now dimeric ligand dimerizes the FGFR [5,6]. In the ‘dimer-of-dimers’ model, two independent complexes of FGF and HS cause the dimerization of the FGFR [7].

The interaction of FGF-2 with HS requires particular patterns of sulphation of the glycosaminoglycan chain [8]. Structural characterization of oligosaccharides isolated from HS or heparin that could bind FGF-2 illustrated the importance of contiguous stretches of the disulphated disaccharide, *N*-sulphoglucosamine-iduronate 2-sulphate, for highest apparent affinity [9,10]. A

pentasaccharide containing only one iduronate 2-sulphate within an *N*-sulphoglucosamine background was suggested to be the minimal binding sequence [11], a contention supported by the contacts made within models of co-crystals of FGF-2 and heparin-derived hexasaccharides [12]. However, almost all studies suggested that only oligosaccharides of degree of polymerization (dp) 10–12 and above were able to bind FGF-2 with high affinity and to support the growth-stimulatory activity of FGF-2 [9–11,13–20].

Binding of FGF-2 to the FGFR is thought to induce receptor dimerization and the activation of its intracellular tyrosine kinase [7,21]. The latter phosphorylates tyrosines in the cytoplasmic domain of the receptor and in the protein adaptor SNT/FRS (FGFR substrate-2/suc1-associated neurotrophic factor target) that subsequently act as docking sites for the initiators of intracellular signalling pathways [22–28]. One of these pathways, that of the mitogen-activated protein kinases (MAPKs), plays a central role in the stimulation of cell proliferation. Phosphorylation of two members of the MAPK family, extracellular signal-regulated kinases 1 and 2, also called p44 and p42, respectively, on Thr^{183/202} and Tyr^{185/204} by a cytoplasmic dual-specificity MAPK kinase, MEK-1, is often associated with cell proliferation [29–31]. Activated p42/44^{MAPK} phosphorylate a range of substrates, including downstream kinases such as p90^{RSK} (ribosomal subunit S6 kinase of 90 kDa, or RSK1).

We have previously shown that in rat mammary fibroblasts a key function of the HS-co-receptor is to enable FGF-2 to induce

Abbreviations used: dp, degree of polymerization; FGF, fibroblast growth factor; FGFR, FGF receptor; HS, heparan sulphate; MAPK, mitogen-activated protein kinase; p90^{RSK}, ribosomal subunit S6 kinase of 90 kDa.

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the sustained dual phosphorylation of MAPK, which is required for cell proliferation [32]. In this study, we examine the relationship between the length of heparin-derived oligosaccharides, their binding kinetics to FGF-2 and the ability of these oligosaccharides to enable the proliferation of chlorate-treated rat mammary fibroblasts induced by FGF-2. Using an optical biosensor approach, the binding parameters of FGF-2 for heparin-derived oligosaccharides of different lengths have been determined for the first time. FGF-2 binds to tetrasaccharides, but as the chain length increases to octasaccharide, there is a substantial increase in association rate constant. As the oligosaccharide length increases to decasaccharides and longer, the association rate constant of FGF-2 reduces substantially. In chlorate-treated rat mammary fibroblasts, even tetrasaccharides are able to restore the growth-promoting activity of FGF-2, though heparin-derived oligosaccharides of $\text{dp} \geq 10$ are more potent, and this is achieved at least in part by enabling FGF-2 to stimulate the sustained dual phosphorylation of $\text{p42/44}^{\text{MAPK}}$. Taken together, these results demonstrate that tetrasaccharides not only bind FGF-2, but that they can be functional partners in the FGF-2 dual-receptor system, which sets important limits on the different models proposed for the FGF-2-HS-FGFR complex.

EXPERIMENTAL

Materials and cells

Human recombinant FGF-2 was produced in-house [33] or obtained from R&D Systems (Abingdon, Oxon, U.K.). Heparin-derived oligosaccharides (dp 4 to dp 26), prepared from partial heparinase I digests of pig mucosal heparin, were obtained from Iduron (Manchester, U.K.); the main disaccharide unit in these saccharides (> 75%) is IdoA,2S-GlcNSO₃,6S (where IdoA is iduronic acid). Porcine intestinal mucosal heparin, trisulphated heparin disaccharide and streptavidin were from Sigma. All reagents for electrophoresis were purchased from Bio-Rad (Hemel Hempstead, Herts., U.K.). PD098059 and biotin-XX-hydrazide were from Calbiochem (Nottingham, U.K.) and NHS(LC)-biotin was from Pierce and Warriner (Chester, U.K.). The antibodies against the phosphorylated forms of p90^{RSK} and against the dually phosphorylated $\text{pThr}^{183/202}/\text{pTyr}^{185/204}$ forms of $\text{p42/44}^{\text{MAPK}}$ were purchased from New England Biolabs (Hitchin, Herts., U.K.). Secondary peroxidase-labelled anti-IgG antibodies were from Amersham Bioscience (Little Chalfont, Bucks., U.K.). The Rama 27 cells are rat fibroblasts derived from normal rat mammary gland and were grown as described previously [32,34]. Northern blotting detected mRNAs that encode FGFR1 in Rama 27 fibroblasts, but not mRNAs encoding other FGFRs, whereas reverse transcriptase PCR experiments have shown a mixture of transcripts encoding the two and three Ig loop isoforms of FGFR1 (results not shown), which is supported by cross-linking [35].

Measurement of DNA synthesis

Sulphated glycosaminoglycan-deficient Rama 27 cells were prepared as described previously [32,36]. Cells were incubated for 4 h in sulphate-free Dulbecco's modified Eagle's medium, supplemented with 10% (v/v) dialysed fetal calf serum and 15 mM NaClO_3 . Following trypsinization, the cells were seeded in 24-well plates in sulphate-free Dulbecco's modified Eagle's medium supplemented with 15 mM NaClO_3 for 24 h. Rama 27 cells were then washed twice in PBS and the culture medium replaced with sulphate-free Dulbecco's modified Eagle's medium supplemented with 15 mM NaClO_3 and 250 $\mu\text{g}/\text{ml}$ BSA. After 24 h

in this medium, FGF-2 and oligosaccharides were added as described in the figure legends. [methyl-³H]Thymidine (40 $\mu\text{Ci}/\text{ml}$, 0.8 μM) was added directly to the culture medium 18 h later for 1 h. The cells were washed twice in PBS, then macromolecules precipitated at 4 °C in 5% (w/v) trichloroacetic acid for at least 30 min, washed in 95% (v/v) ethanol, air dried for 20 min and finally solubilized in 0.1 M NaOH for 30 min at 37 °C. The radioactivity incorporated into DNA was measured by liquid scintillation counting.

Identification of phosphorylated forms of $\text{p42/44}^{\text{MAPK}}$ and p90^{RSK} by Western blotting

Sulphated glycosaminoglycan-deficient Rama 27 cells were seeded at equal densities in 10 cm-diameter culture dishes, and then treated identically as for the DNA synthesis assay up to and including the addition of growth factors. At the times indicated in the figure legends, cells were washed twice with ice-cold PBS and then lysed in 300 μl of lysis buffer [50 mM Tris/HCl, pH 6.8, 1% (w/v) SDS, 0.006% (w/v) Bromophenol Blue, 2% (v/v) β -mercaptoethanol, 10% (v/v) glycerol and protease inhibitor cocktail (Complete, Roche)], scraped with a rubber policeman and collected in 1.5 ml Eppendorf tubes. All steps were performed at 4 °C. Identical amounts of protein were separated by SDS/PAGE. After transfer to nitrocellulose, the membranes were blocked for 30 min in blotting solution [Tris-buffered saline containing 5% (w/v) non-fat dry milk and 0.05% (v/v) Tween 20]. Incubation with primary antibodies diluted at 1:500 was carried out overnight at 4 °C in the blotting solution. After five washes in Tris-buffered saline containing 0.05% (v/v) Tween 20, the nitrocellulose membrane was incubated with secondary peroxidase-conjugated antibodies to IgG antibodies, diluted 1:1000 in the blotting solution. After several washes with 0.05% (v/v) Tween 20 in Tris-buffered saline, immunoreactive proteins were revealed with the SuperSignal chemiluminescent detection system (Perbio, Chester, U.K.) on Hyperfilm (Amersham Bioscience).

Biotinylation of oligosaccharides

Porcine intestinal mucosal heparin was biotinylated on amino groups with NHS(LC)-biotin, and free biotin was removed by gel-filtration chromatography, as described [37]. Biotinylation of oligosaccharides (1 mg/ml in PBS) at their reducing end was performed by addition of sufficient 50 mM biotin-XX-hydrazide in DMSO to give a final concentration of 5 mM. Samples were securely capped, mixed end-over-end for 2 h at room temperature and were diluted with an equal volume of water. Oligosaccharides of $\text{dp} \geq 10$ were applied to a 0.5 ml column of DEAE-Sepharcel equilibrated with 50 mM NaCl/10 mM phosphate buffer, pH 7.0. After washing extensively with the same solution to remove unreacted biotin reagent, the bound oligosaccharides were eluted with 1 ml of 1.5 M NaCl. Oligosaccharides from dp 4 to dp 8 were diluted with water to a volume of 1 ml and injected on to a Hypersil strong anion-exchange HPLC column (250 mm $\times$ 4.6 mm, 5 μm pore; ThermoQuest, Runcorn, Cheshire, U.K.) monitored by UV absorbance at 232 nm. The column was washed with water followed by 0.1 M NaCl to remove unreacted biotin reagent. Bound oligosaccharides were eluted with a 1 M NaCl step. Successful biotinylation of oligosaccharides was confirmed by a dot-blot. Samples were diluted 10-fold with water and spotted on to a DEAE-membrane. The membrane was washed with water, blocked with half-strength Superblock (Pierce and Warriner) plus 0.1% (v/v) Tween 20 for

2 h and then incubated with a 1:5000 dilution of streptavidin–horseradish peroxidase conjugate in quarter-strength Superblock plus 0.1 % (v/v) Tween 20 for 30 min. The membrane was washed with five changes (each 5 min) of 50 mM NaCl/10 mM Na_2HPO_4 , pH 7.0 and bound horseradish peroxidase was detected by ECL (Amersham Bioscience).

Immobilization of oligosaccharides

Streptavidin was immobilized on planar aminosilane surfaces according to the recommendations of the manufacturer (Affinity Sensors, Cambridge, U.K.). Controls showed that in PBST [PBS supplemented with 0.02 % (v/v) Tween 20] FGF-2 did not bind to this surface or to a streptavidin surface derivatized with biotin hydrazide (result not shown). Biotinylated heparin was immobilized as described [37]. For the oligosaccharide surfaces, each size of oligosaccharide was immobilized to yield a response of no more than 100 arc s (600 arc s corresponds to 1 ng of protein/ mm^2 of the cuvette surface) in the presence of 2 $\mu\text{g}/\text{ml}$ FGF-2. The distribution of the bound FGF-2, and by inference the immobilized heparin or oligosaccharide, on the surface of the biosensor cuvette was inspected by examination of the resonance scan in the course of the association phase of the binding reactions. Resonance scans showed that at all times FGF-2 was distributed uniformly on the sensor surface and therefore was not microaggregated (results not shown).

Competitive-binding assays

Heparin-derivatized aminosilane surfaces and a customized program for the Auto+instrument (available from the manufacturer, Affinity Sensors) were used in these experiments. The cuvette was equilibrated in 40 μl of PBST and then 5 μl of the relevant dilution of an oligosaccharide in PBST was added, followed by 5 μl of 10 $\mu\text{g}/\text{ml}$ FGF-2 to initiate binding. The binding reaction was continued for 5 min. The surface was washed three times with 50 μl of PBST and 2 min later was regenerated with 2 M NaCl. The reagents were stored in the instrument's reagent trays at 4 °C for the duration of the experiments. The heparin-binding capacity of FGF-2 remained intact in the course of the experiment, since no change was detected in the amount of FGF-2 bound to immobilized heparin in the absence of competing polysaccharide at the start and the end of the experiments (results not shown). The extent of binding was calculated by fitting the association curve to a single-site binding model using the non-linear curve fitting FastFit software (Affinity Sensors).

Measurement of binding kinetics

Binding reactions were carried out as described in [36,38] with minor modifications. A single binding assay consisted of adding the FGF-2 at a known concentration in 5 μl of PBST to a cuvette containing 45 μl of PBST. The association reaction was followed over a set time, usually 210 s. The cuvette was then washed three times with 50 μl of PBST and the dissociation of bound ligate into the bulk PBST was followed over time. The surface was regenerated by washing twice with 50 μl of 2 M NaCl/10 mM Na_2HPO_4 , pH 7.2. Binding parameters were calculated from the association and dissociation phases of the binding reactions using the non-linear curve fitting FastFit.

A single binding assay yielded four binding parameters: the slope of the initial rate of association, the on-rate constant (k_{on}) and the extent of binding, all calculated from the association phase, and the off-rate constant (k_{off} , equivalent to the dissociation rate constant, k_{diss}), calculated from the dissociation phase.

Data analysis

In kinetic experiments, k_{on} was only determined at low concentrations of FGF-2, whereas k_{off} was measured at higher concentrations of FGF-2 with competing heparin (100 $\mu\text{g}/\text{ml}$) in the dissociation buffer to avoid any rebinding artifacts [39,40]. The association rate constant (k_{ass}) was calculated from the values of k_{on} determined at eight concentrations of FGF-2. The equilibrium dissociation constant (K_{d}) was then calculated from the kinetic parameters as $k_{\text{diss}}/k_{\text{ass}}$, and, independently, from the extent of binding observed at equilibrium.

Analysis of binding kinetics in optical biosensors may generate artifactual second-phase binding sites, due either to rates of diffusion of soluble ligate approaching or exceeding the rate of association, or to steric hindrance at the binding surface [41,42]. Three steps were taken to avoid these two possible artifacts. First, since the oligosaccharides were immobilized through their reducing ends, immobilization was oriented rather than random, thus reducing steric hindrance between the surface and the oligosaccharide. Secondly, concentrations of FGF-2 were kept as low as possible. Thirdly, for each surface used to measure kinetics, a plot of the initial rate constant against FGF-2 concentration was examined, to ensure that the points fitted a straight line with a positive slope, which indicates an absence of diffusion-limited association [43]. A single-site binding model fitted the data at least as well as a two-site binding model in both the competitive binding assays and the kinetic experiments. Therefore the binding reaction between FGF-2 and the polysaccharides was deemed to be monophasic and a single-site model was used to calculate all binding parameters.

RESULTS

Interaction of FGF-2 with oligosaccharides

Competition by oligosaccharides for FGF-2 binding to heparin

In an initial set of experiments, a competition assay was used in which soluble oligosaccharides of different lengths competed with heparin, immobilized on the sensor surface, for the binding of FGF-2 (Figure 1). Disaccharides (dp 2) in solution failed to inhibit the binding of FGF-2 to the heparin immobilized on the biosensor cuvette (Figure 1), even at concentrations as high as 1.7 mg/ml (result not shown). In contrast, tetrasaccharides competed efficiently for FGF-2 binding to the immobilized heparin. The concentration of dp 4 required to inhibit 50 % of the binding of FGF-2 to heparin (IC_{50}) was $0.8 \pm 0.3 \mu\text{g}/\text{ml}$ (mean $\pm$ S.D., four experiments), and 10 $\mu\text{g}/\text{ml}$ dp 4 virtually abolished the binding. Surprisingly, the longer oligosaccharides had similar IC_{50} values ($n = 4$: dp 6, $0.6 \pm 0.3 \mu\text{g}/\text{ml}$; dp 8, $1 \pm 0.6 \mu\text{g}/\text{ml}$; dp 10, $0.7 \pm 0.1 \mu\text{g}/\text{ml}$; dp 12, $0.7 \pm 0.2 \mu\text{g}/\text{ml}$; dp 14, $0.3 \pm 0.1 \mu\text{g}/\text{ml}$) and also maximally inhibited FGF-2 binding to heparin at 10 $\mu\text{g}/\text{ml}$ (Figure 1). Whereas the IC_{50} value for heparin ($0.7 \pm 0.2 \mu\text{g}/\text{ml}$) was similar to that of the oligosaccharides, the inhibition curve for heparin was much steeper and consequently 1 $\mu\text{g}/\text{ml}$ heparin was sufficient to abolish FGF-2 binding to the immobilized heparin. These results are in marked contrast to those obtained with hepatocyte growth factor/scatter factor, where the IC_{50} decreased progressively as the number of disaccharide units increased [44].

Kinetics of FGF-2 binding to oligosaccharides

The kinetics of the interaction of FGF-2 with reducing-end-biotinylated oligosaccharides immobilized on a streptavidin-derivatized cuvette were determined in an optical biosensor. In

Figure 1 Competition of FGF-2 binding to immobilized heparin by heparin oligosaccharides of different lengths

The extent of binding of FGF-2 to heparin immobilized on an aminosilane surface was measured in the presence of increasing concentrations of oligosaccharides of different lengths (see the Experimental section). The amount of FGF-2 bound to the immobilized heparin at each concentration of oligosaccharide was calculated from the binding curve by non-linear curve fitting using a one-site binding model (see the Experimental section). Errors for individual datum points generated by the curve fitting were less than 1% of the mean and were omitted for clarity. Results are expressed as a percentage of the maximal binding of FGF-2 to heparin (145 arc s) observed in the absence of competing oligosaccharides. Similar results obtained in four separate experiments were used to calculate the IC_{50} values reported in the text.

all cases, the binding of FGF-2 to the oligosaccharides was monophasic and there was no evidence for biphasic association kinetics. Thus the distribution of the data points around a one-site model was random and within the limits of instrument noise (Figures 2A–2H for dp 4, not shown for other oligosaccharides). Moreover, the plot of the slope of the initial rate against concentration of FGF-2 was linear, indicating that binding was not limited by diffusion (Figure 2I for dp 4, not shown for other oligosaccharides). The association rate constant (k_{ass}) of FGF-2 for tetrasaccharides was relatively fast, $560\,000\text{ M}^{-1}\cdot\text{s}^{-1}$. An increase in length of one disaccharide unit to dp 6 resulted in a near doubling the k_{ass} to $950\,000\text{ M}^{-1}\cdot\text{s}^{-1}$ and a further increase by one disaccharide to dp 8 resulted in k_{ass} again doubling to $2\,000\,000\text{ M}^{-1}\cdot\text{s}^{-1}$. However, further increases in the length of the oligosaccharides, to dp 10 and dp 14, resulted in a reduction in the association rate constant, such that the k_{ass} of FGF-2 for dp 14 was similar to that for dp 4 (Table 1). The dissociation rate constant (k_{diss}) was measured in a second, independent set of experiments, in which $100\text{ }\mu\text{g/ml}$ heparin was included in the dissociation buffer to prevent re-binding [39,40]. Overall, k_{diss} did not vary appreciably with the length of the oligosaccharides, although there was a general trend of a reduction in k_{diss} as oligosaccharide length increased. Therefore, the equilibrium

Figure 2 Monophasic binding of FGF-2 to immobilized heparin tetrasaccharides

The binding kinetics of FGF-2 to immobilized tetrasaccharides was measured as described in the Experimental section. (A–H) The distribution of the data points (jagged line) around a one-site binding model (horizontal line at 0 arc s) is shown for each of the concentrations of

FGF-2 used in the binding assay. Instrument noise was ± 0.5 arc s. Concentrations of FGF-2 were: A, 5.6 nM; B, 8.3 nM; C, 11 nM; D, 17 nM; E, 22 nM; F, 33 nM; G, 42 nM; H, 56 nM. (I) A plot of the slope of the initial rate of association against concentration of FGF-2.

Table 1 Kinetics of FGF-2 binding to immobilized oligosaccharides of different lengths

Binding parameters were determined in an optical biosensor (see the Experimental section). The S.E.M. of the k_{ass} was derived from the deviation of the data from a one-site binding model, calculated by matrix inversion using the FastFit software provided with the instrument. No evidence was found for a two-site model of association and so the FGF-2-binding sites in each length of oligosaccharide were homogenous in this respect. At least four independent sets of k_{on} were measured and the four resulting values for k_{ass} and their errors were combined. The correlation coefficient of the linear regression through the k_{on} values is represented by r . The k_{diss} is the mean $\pm$ S.E.M. of at least five values, obtained at high concentrations of FGF-2 in the presence of competing heparin. No evidence was found for a two-site model of dissociation and so the FGF-2-binding sites in each length of oligosaccharide were homogenous in this respect as well. The K_{d} (kinetic) was calculated from the ratio of $k_{\text{diss}}/k_{\text{ass}}$ and its S.E.M. is the combined S.E.M. of the two kinetic parameters. The K_{d} (equilibrium) was calculated from the extent of binding at or near equilibrium and its S.E.M. is the combined S.E.M. of four independent determinations of K_{d} .

Length of oligosaccharide	k_{ass} ($\text{M}^{-1} \cdot \text{s}^{-1}$)	r	k_{diss} (s^{-1})	K_{d} (kinetic) (nM)	K_{d} (equilibrium) (nM)
dp 4	$564\,000 \pm 240\,000$	0.922	0.035 ± 0.005	62 ± 28	77 ± 9
dp 6	$952\,000 \pm 340\,000$	0.927	0.026 ± 0.002	27 ± 10	71 ± 9
dp 8	$2\,000\,000 \pm 610\,000$	0.941	0.022 ± 0.002	11 ± 3	11 ± 2
dp 10	$1\,000\,000 \pm 190\,000$	0.979	0.028 ± 0.003	28 ± 6	18 ± 2
dp 14	$470\,000 \pm 160\,000$	0.909	0.020 ± 0.002	42 ± 15	30 ± 6

dissociation constant (K_{d}) of the interaction between FGF-2 and the oligosaccharides was determined primarily by the k_{ass} . The highest-affinity interaction was that between FGF-2 and dp 8, with the shorter and longer oligosaccharides possessing a lower affinity for FGF-2 (Table 1 and Figure 2). The K_{d} of FGF-2 for the oligosaccharides was also calculated from the amount of FGF-2 binding observed near equilibrium. These correspond to the values determined from the kinetic parameters, with the exception of the value for hexasaccharides, which was 2-fold higher (Table 1). The binding of FGF-2 to the immobilized oligosaccharides was monophasic, was clearly not limited by diffusion and the kinetic and equilibrium determinations of affinity are reasonably self-consistent. Therefore, the binding parameters calculated from these experiments (Table 1) are likely to reflect the intrinsic values for these interactions. A considerable body of evidence shows that some oligosaccharides and HS chains that bind FGF-2 inhibit, rather than promote, the activity of the growth factor [13,14,36,45]. It was therefore important to establish whether the heparin-derived oligosaccharides that bound FGF-2 (Table 1) inhibited or promoted FGF-2-dependent cell proliferation.

Effect of oligosaccharides on FGF-2 stimulation of DNA synthesis

As described previously [32,36], FGF-2 is a strong mitogen for Rama 27 fibroblasts and this effect is strictly dependent upon HS. Thus FGF-2 does not stimulate DNA synthesis in chlorate-treated Rama 27 fibroblasts, which are deficient in sulphated HS. However, the addition of purified endogenous HS or heparin restores the growth-stimulatory activity of FGF-2 [32,36]. Therefore we examined the ability of heparin-derived oligosaccharides of different lengths to restore the growth-stimulatory activity of FGF-2 in chlorate-treated Rama 27 fibroblasts. Oligosaccharides of dp 2 to dp 24 were added simultaneously with the growth factor to chlorate-treated cells. Whereas 10 ng/ml is the optimal concentration of heparin in this system, studies in other systems suggest that oligosaccharides need to be used at higher concentrations and that in general only oligosaccharides of dp ≥ 10 promote the growth-stimulatory activity of FGF-2 [10,16,19]. Surprisingly, at 10 ng/ml, all the oligosaccharides of dp ≥ 4 enabled FGF-2 to stimulate a maximal level of DNA synthesis, although disaccharides were inactive (Figure 3A). When the dose-dependence of the activity of the saccharides was titrated, it was found that a concentration of around 5 ng/ml of dp 4, dp 6 or dp 8 was required to elicit a half-maximal response (EC_{50}), whereas the longer oligosaccharides (dp ≥ 10) had an EC_{50} of 0.2–0.5 ng/ml, and so were about 10-fold more potent (Figure 3B). Therefore, in this assay the oligosaccharides fall

into three groups according to their potency, and there is not a gradation between their length and their potency. Importantly, these results show that all the oligosaccharides that bind FGF-2 are able to restore the growth-stimulatory activity of FGF-2 in chlorate-treated cells.

Effect of oligosaccharides on FGF-2 stimulation of the phosphorylation of p42/44^{MAPK}

We have previously shown that the addition of purified HS or of heparin to chlorate-treated fibroblasts was required to allow the sustained dual phosphorylation of p42/44^{MAPK}, which is itself required for the stimulation of DNA synthesis [32]. We therefore determined whether the heparin-derived oligosaccharides were similarly able to restore the sustained dual phosphorylation of p42/44^{MAPK} in chlorate-treated cells. FGF-2 and oligosaccharides were used at concentrations that resulted in a maximal growth-stimulatory response (10 ng/ml). As shown in Figure 4(A), FGF-2 alone only stimulates a transient dual phosphorylation of p42/44^{MAPK} in chlorate-treated cells. In this case, the dual phosphorylation of p42/44^{MAPK} is apparent after 5 min and then reaches maximal levels at 15 min, after which it falls to near basal levels by 30 min, as described previously [32]. In the presence of disaccharides, which do not allow FGF-2 to stimulate DNA synthesis in chlorate-treated cells (Figure 3), the stimulation of dual phosphorylation of p42/44^{MAPK} by FGF-2 remains transient (Figure 4A). However, in the case of those oligosaccharides which enable FGF-2 to stimulate cell proliferation, such as the tetrasaccharides, the FGF-2 stimulated dual phosphorylation of p42/44^{MAPK} was apparent after 5 min, reached a peak at 15 min and then declined to a plateau by 30 min (Figure 4A). The plateau, which corresponds to about half the maximal level of dual phosphorylation observed, represents the sustained response that is necessary for the stimulation of DNA synthesis [32]. All the oligosaccharides from dp 4 to dp 14 allowed FGF-2 to elicit the same sustained kinetics of dual phosphorylation of p42/44^{MAPK} (Figure 4A). The sustained dual phosphorylation of p42/44^{MAPK} stimulated by FGF-2 in chlorate-treated cells in the presence of the oligosaccharides also resulted in a sustained phosphorylation of p90^{RSK}, a target of active p42/44^{MAPK} (Figure 4B for dp 4 and 12). In contrast, the oligosaccharides alone only stimulated a weak but detectable transient dual phosphorylation of both p42/44^{MAPK} and p90^{RSK} (Figure 4B for dp 2, dp 4 and dp 12). This activity has been observed previously with heparin and it has been ascribed to the activation of a cellular receptor for heparin [32]. Therefore, these results demonstrated that in chlorate-treated Rama 27 cells,

Figure 3 Stimulation of DNA synthesis in chlorate-treated Rama 27 fibroblasts by FGF-2 in the presence of increasing concentrations of heparin oligosaccharides

(A) FGF-2 and heparin oligosaccharides (both 10 ng/ml) were added to serum-starved chlorate-treated Rama 27 fibroblasts (see the Experimental section). Control (ctrl) received no additions. In a separate experiment, FGF-2 stimulated the incorporation of 7527 ± 376 c.p.m. of [^3H]thymidine into the DNA of untreated serum-starved cells. Results are the means $\pm$ S.D. of triplicate wells of one of four experiments. (B) Dose-response of active heparin oligosaccharides of different lengths added with FGF-2 (10 ng/ml). Results are the mean of triplicate wells of one of four experiments. The S.D. was less than 10% of the mean and is omitted for clarity in (B).

heparin-derived oligosaccharides as short as dp 4 are able to restore the growth-stimulatory activity of FGF-2 and this is achieved, at least in part, by enabling a sustained phosphorylation of p42/44^{MAPK}.

DISCUSSION

FGF-2 possesses a dual receptor system consisting of tyrosine kinase receptors (FGFR) and HS proteoglycans, and both receptors must be present for FGF-2 to stimulate cell proliferation. The interaction of FGF-2 with HS/heparin depends on specific patterns of sulphation and the presence of iduronic acid [9,11,17,19]. To shed light on the reason for the dependence of FGF-2-mediated cell proliferation on HS/heparin we have rigorously analysed the relationship between the length of heparin-derived oligosaccharides and their ability to bind FGF-2 and to promote FGF-2-mediated DNA synthesis in chlorate-treated Rama 27 cells.

Previous studies have suggested that the minimal binding structure in heparin/HS that could interact with FGF-2 was a tetra- or pentasaccharide [11,12,45,46], with maximal affinity being attained around a dp of 12–14 (e.g. [9,10]). However, in these previous studies, qualitative techniques (affinity chromatography, filter binding, etc.) were used to measure the interaction between FGF-2 and the oligosaccharide, in which 'affinity' was equated with the concentration of NaCl required to elute the oligosaccharide from the protein. In the present study the oligosaccharides (or heparin) are immobilized on streptavidin via a approx. 2.5 nm spacer (which is greater than the radius of the FGF-2 protein), and the B_{max} of FGF-2 on each oligosaccharide-derivatized surface (results not shown) indicates that there is less than one oligosaccharide immobilized for every three streptavidin molecules. Therefore the biosensor-based assay used here is much closer to an optimal binding geometry than assays used in previous studies. This may explain why both the competition assay and the kinetic studies (Figure 1 and Table 1) demonstrate

Figure 4 Stimulation of the dual phosphorylation of p42/44^{MAPK} and p90^{RSK} by FGF-2 in chlorate-treated Rama 27 fibroblasts

(A) Time course of dual phosphorylation of p42/44^{MAPK} following addition of FGF-2 and oligosaccharides of different lengths. Rama 27 fibroblasts were stimulated with FGF-2 (10 ng/ml), heparin oligosaccharides of different lengths (10 ng/ml) for 0–60 min as indicated. Activated p42/44^{MAPK} were detected by Western blotting with a phosphospecific antibody directed against dually phosphorylated (Thr¹⁸³/Tyr¹⁸⁵/Tyr²⁰⁴) p42/44^{MAPK} (see the Experimental section). (B) Time course of p42/44^{MAPK} and p90^{RSK} phosphorylations following exposure to FGF-2 and selected oligosaccharides at 10 ng/ml. Phosphorylated p90^{RSK} was detected by Western blotting as described in the Experimental section.

unequivocally that oligosaccharides of $dp \geq 4$ all bind FGF-2, and that the affinity of FGF-2 for tetrasaccharides is very similar to that for tetradecasaccharides, which were previously thought to represent the high-affinity binding species. Interestingly, in co-crystals of FGF-2 and heparin-derived oligosaccharides, a tetrasaccharide is accommodated by the major heparin-binding site of FGF-2 [12]. The increase in k_{ass} observed in the sequence $dp\ 4$ – $dp\ 6$ – $dp\ 8$ indicates that as the oligosaccharide increases in length by successive disaccharide units, the probability of a molecular collision and productive binding between FGF-2 and the oligosaccharide resulting in a binding reaction also increases. This result is in accordance with the notion that, in solution, the structure of a heparin oligosaccharide approximates an anisotropic rod [47,48]. However, the reduction in k_{ass} observed with oligosaccharides of $dp \geq 10$ was unexpected. This result is unlikely to be due to an artifact of the binding assay, since the binding reactions were resolutely monophasic regardless of the length of the oligosaccharides. Studies of the flexibility of the heparin chain indicate that even a single glucuronate introduces considerable flexibility [47,48] and that sulphated oligosaccharides may also have some flexibility around the anomeric carbon [49]. Therefore, an explanation of the reduction in k_{ass} observed as the chain length increases from $dp\ 10$ may be that the glycosidic bonds of these longer oligosaccharides possess considerable flexibility, as suggested by a model of the crystal structure of a complex of FGF-1–heparin decasaccharide–FGFR1 [6]. The consequence of the increased conformational variability of the longer oligosaccharides is that the probability of a productive molecular collision between FGF-2 and the oligosaccharide is reduced and so k_{ass} is lower.

In chlorate-treated Rama 27 cells, the shortest heparin-derived oligosaccharides that are able to restore the growth-stimulatory activity of FGF-2 are tetrasaccharides. While the oligosaccharides of $dp \leq 8$ were less potent (EC_{50} of 5 ng/ml) than the oligosaccharides of $dp \geq 10$ (EC_{50} of 0.2–0.5 ng/ml), in terms of concentration they are nevertheless very active, since 5 ng/ml tetrasaccharide corresponds to approx. 5 nM. This result concurs with just one previous study in which heparin trisaccharides were observed to restore the stimulation of cell proliferation by FGF-2 in HS-deficient cells [50]. All other studies have found that longer oligosaccharides were necessary for full activity (e.g. [10,13–15,19,20]). These results, coupled to previous studies using qualitative binding techniques, have led to a consensus view that only the longer oligosaccharides of $dp \geq 10$ are able to support the growth-stimulatory activity of FGF-2. The apparent discrepancy between the present results and this consensus view is, however, readily resolved and reinforces the contention that short oligosaccharides, e.g. $dp\ 4$, derived from heparin, but not HS, represent the smallest active unit.

Oligosaccharides derived from the sulphated domains of HS by digestion with heparinase III will have a similar content of N-sulphated glucosamine and iduronic acid (including a substantial 2-O-sulphated iduronate content) to similarly sized oligosaccharides derived from heparin. However, sequencing of such HS-derived oligosaccharides has revealed that they are sparingly 6-O-sulphated and a significant number are completely deficient in 6-O-sulphate groups. For example, only 12% of sequenced hexasaccharides and 29% of sequenced octasaccharides from 3T3 fibroblast-derived HS contained a 6-O sulphate group [51]. In contrast heparin oligosaccharides will be substantially 6-O-sulphated. The 6-O sulphate groups are clearly not required for FGF-2 binding [9,11,16], but are likely to be essential for the growth-stimulatory activity of FGF-2 [13,16,18,20], probably by mediating an interaction with the FGFR [7,52,53]. Therefore, it seems likely that these differences in 6-O sulphate

content explain why heparin-derived tetrasaccharides enable FGF-2-stimulated cell proliferation, whereas only HS-derived oligosaccharides of $dp \geq 10$ are active [19,20].

In the case of heparin-derived oligosaccharides, some activity has previously been noted for oligosaccharides as short as $dp\ 4$ [13] and $dp\ 6$ [10,15], although full activity was only observed with a $dp \geq 12$. The relative lower activity of the oligosaccharides of $dp\ 4$ – $dp\ 10$ observed in these studies [10,13,15] compared with the present study may reflect the use of sub-optimal concentrations of FGF-2 (1.8 ng/ml to 2 ng/ml). Moreover, the complement of FGFR types expressed by the cells used in these other studies may be different to that on the Rama 27 fibroblasts. Since different FGFRs may have different oligosaccharide requirements [14], this may also contribute to the relative differences in activity observed for small saccharides.

A more critical discrepancy may be that we observe the transition from lower-activity oligosaccharides to higher-activity oligosaccharides between $dp\ 8$ and $dp\ 10$ (Figure 3), whereas in other studies [10,13,15] this transition is observed at one disaccharide unit longer, between $dp\ 10$ and $dp\ 12$. This may be due to the methods used to generate the oligosaccharides. Thus the oligosaccharides used in the present study are generated by heparinase I digestion and therefore have the expected N-sulphoglucosamine at the reducing end, though the non-reducing-end uronate will be $\Delta 4,5$ -unsaturated. Therefore these approximate to the expected native structure in overall charge distribution and ring structure. However, the other studies have produced oligosaccharides by nitrous acid cleavage followed by reduction of the reducing end anhydromannose to anhydromannitol. Consequently, these oligosaccharides are essentially an N-sulphoglucosamine short compared with the native structure. Therefore the activity of short oligosaccharides would appear to have been overlooked in previous studies, due in part to the choice of concentration of FGF-2, whereas the method of cleavage of heparin is likely to account for the single disaccharide unit difference in the transition from lower- to higher-activity oligosaccharides. It is interesting to note that oligosaccharides as short as $dp\ 4$ have also been shown to be sufficient for the growth-stimulatory activity of hepatocyte growth factor/scatter factor [44].

The activity of the oligosaccharides of $dp\ 4$ to $dp\ 8$ is clearly less than that of their longer counterparts. Therefore, the question arises whether such small oligosaccharides enable FGF-2 to activate the FGFR and downstream signals in the same way as HS/heparin, or whether the small oligosaccharides operate by a different mechanism that may only function in a restricted number of cell systems. Previously, we have demonstrated that the activation of the MAPK pathway, which is required for the stimulation of cell proliferation by FGF-2 and that lies downstream of the FGFR, is regulated by HS/heparin. Thus in the absence of HS/heparin, FGF-2 only stimulated a transient phosphorylation of $p42/44^{\text{MAPK}}$, which was insufficient for the later stimulation of DNA synthesis. In contrast, in the presence of HS/heparin, FGF-2 stimulated a sustained phosphorylation of $p42/44^{\text{MAPK}}$, which enabled the stimulation of DNA synthesis [32]. The present results (Figure 4) show clearly that all the active oligosaccharides ($dp\ 4$ to $dp\ 14$) have the same effect on the dual phosphorylation of $p42/44^{\text{MAPK}}$: they allow FGF-2 to promote a sustained phosphorylation of $p42/44^{\text{MAPK}}$. This result clearly suggests that the short oligosaccharides of $4 \leq dp \leq 8$ function in the same way as the longer oligosaccharides of $dp \geq 10$, which most previous studies have found to be the active species. Therefore, it appears that a common mechanism underlies the activation of the FGFR, by FGF-2 and oligosaccharides of $dp \geq 4$, leading to downstream signals such as $p42/44^{\text{MAPK}}$.

It is interesting that the observed ranking of the oligosaccharides, in terms of potency in the DNA synthesis assays ($\text{dp } 4 \approx \text{dp } 6 \approx \text{dp } 8 < \text{dp } 10 \approx \text{dp } 12 \approx \text{dp } 24$; Figure 3), does not correlate with either the association rate constant or the affinity of the interaction of FGF-2 with the oligosaccharides. The absence of any such correlation suggests that the interaction of FGF-2 with the oligosaccharides is not rate limiting with respect to the activated FGFR generating the intracellular signals, e.g. sustained dual phosphorylation of $\text{p42/44}^{\text{MAPK}}$ required for cell proliferation. However, the oligosaccharides are absolutely necessary for the generation of such signals. Since the early peak of phosphorylation of $\text{p42/44}^{\text{MAPK}}$ is observed in the absence of oligosaccharides, then the FGF–FGFR interaction may similarly not be rate limiting. This leaves the interaction of the oligosaccharides with another molecule, e.g. FGFR, as a rate-limiting step. Such an interaction may be facilitated by the increased flexibility of oligosaccharides of $\text{dp} \geq 10$. However, an alternative explanation is that binding and assembly of the ligand–receptor complex is a rapid process. Therefore, the rate-limiting step for the generation of the downstream signals required for cell proliferation, which depends on the presence of oligosaccharides of $\text{dp} \geq 4$ and which is more efficient with oligosaccharides of $\text{dp} \geq 10$, may be a post-receptor binding event, such as receptor trafficking and down-regulation.

The present results can be used to test certain aspects of the three models of the FGF–HS–FGFR complex, the growth hormone [3,4], HS-dependent dimerization [5,6] and the dimer-of-dimers [7] models. For steric reasons, FGF-2 (diameter approx. 4.5 nm) requires an oligosaccharide of at least $\text{dp } 10$ for dimerization in *cis* mode [54]. Therefore, since tetrasaccharides allow FGF-2 to stimulate cell proliferation, a model where FGF-2 is dimerized in *cis* mode on HS/heparin is unlikely. Dimerization in *trans* mode remains a possibility, although with small oligosaccharides this would preclude any additional interactions between the oligosaccharide and the FGFR, for which there is considerable evidence [52,53]. Moreover, the observation that covalent 1:1 complexes of a HS dodecasaccharide and FGF-2 possessed normal biological activity also argues against such dimerization models [20]. In addition, the dimerization model is inconsistent with the observation that FGF-2 is able to elicit HS-independent transient phosphorylation of $\text{p42/44}^{\text{MAPK}}$. In contrast, the growth hormone and the dimer-of-dimers models are both consistent with the present data. However, these models are static and there is a possibility that the rate-limiting step that depends on the oligosaccharides occurs after the formation of the functional receptor–ligand complex. Therefore the answer to the question of why FGF-2 requires HS/heparin to stimulate cell proliferation may not lie in the molecular interactions that underpin the formation of the FGF–FGFR–HS complex, but in subsequent events, as is suggested by other work [55,56].

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