Heparan sulfate (HS) regulates the kinetics of fibroblast growth factor (FGF) stimulated intracellular signaling and differentially activates cell proliferation of cells expressing different FGF receptors (FGFRs). Evidence suggests that HS interacts with both FGFs and FGFRs to form active ternary signaling complexes. Here we compare the interactions of two FGFRs with HS. We show that the ectodomains of FGFR1 Ile and FGFR2 Ile exhibit specific interactions with different characteristics for both heparin and porcine mucosal HS. These glycans are both known to activate FGF signaling via these receptors. FGFR2 interacts with a higher apparent affinity than FGFR1 despite both involving 6-O-, 2-O-, and N-sulfates. FGFR1 and FGFR2 bind heparin with mean association rate constants of 1.9 × 10^5 and 2.1 × 10^6 M^-1 s^-1, respectively, and dissociation rate constants of 1.2 × 10^-2 and 2.7 × 10^-2 s^-1, respectively. These produced calculated affinities of 63 and 13 nm, respectively. Hence, FGFR1 and FGFR2 bind to heparin chains with markedly different kinetics and affinities. We propose a mechanistic model where the kinetic parameters of the HS/FGFR interaction are a key element regulating the formation of ternary complexes and the resulting FGF signaling outcomes.

Fibroblast growth factors (FGFs) utilize a co-receptor system consisting of tyrosine kinase receptors (FGFRs) and heparan sulfate proteoglycans (HSPGs) (1, 2). The FGFRs belong to a family of five genes (FGFR1–5), from which alternative splicing generates diverse isoforms (3, 4). Heparan sulfate (HS) is a family of linear polysaccharides located at the surface of cells and in the extracellular matrix. HS chains are attached to core proteins, forming a class of glycoproteins called proteoglycans (5). HS consists of a backbone disaccharide repeat of alternating glucosamine and hexuronate monosaccharides on which are superimposed highly variable patterns of N- and O-linked sulfate groups and uronate epimerization; that is, iduronate (IdoUA) or glucuronate residues. This creates diverse molecular motifs, which present unique displays of sulfate, carboxyl, and hydroxyl groups (6). The modifications are clustered, producing the domain structure of HS; that is, stretches of sulfated saccharides (termed NS domains or S domains) separated by N-acetyl-rich saccharides (termed NA domains). The NS domains are rich in IdoUA and the NA domains in glucuronate. Heparin can be regarded as a special class of HS expressed by mast cells and also oligodendrocyte type-2 astrocyte progenitor cells (7). Heparin is highly sulfated, contains a high abundance of IdoUA, and lacks the ordered domain structure seen in HS (8). The sequences within heparin may in many aspects resemble those of highly sulfated NS domains of HS.

It has been shown that heparin and HS bind to proteins and modulate their activities via specific sulfated sequences within the chains (commonly within the NS domains) (6, 8). This is particularly apparent for FGFs, which recognize different structural motifs in HS (9–11). It is known that there is some selectivity in many FGF-FGFR interactions (12). HS has been shown to provide an additional level of selectivity, determining which FGFs bind and activate particular FGFRs (13–20). A number of mechanisms by which the co-receptor system functions to transduce an FGF signal have been proposed involving ligand-induced receptor oligomerization (3, 21). First, it has been suggested that HS increases the affinity of FGF2 for FGFRs by altering the conformation of the FGF (2). However, a change in the conformation of FGF2 on binding HS is not seen in structures of co-crystals of FGF2 and heparin-derived oligosaccharides (22). Second, HS has been proposed to dimerize FGFs, thereby facilitating receptor dimerization (23, 24). Finally, HS has been proposed to increase the affinity of FGF2 for FGFRs (25–27), possibly by reducing the dissociation rate constant through the formation of a ternary complex (25). Evidence that HS also interacts directly with FGFRs (14, 28, 29) supports a model where FGFs and FGFRs interact with distinct sites within HS chains to drive the formation of a ternary complex (11, 27, 28).

FGF2 interacts with high affinity with FGFRs in the absence of HS (Kd ~ 10^-10 M) (25, 30, 31). Such interactions can induce receptor activation and transient phosphorylation of p42/44MAPK and p90RSK but not degradation of IKB proteins or cell proliferation (26, 32, 33). The latter only occur in the presence of active heparin or HS and are accompanied by a sustained phosphorylation of p42/44MAPK and p90RSK (33). The kinetics of ternary complex formation, therefore, may be important in determining the time scale of intracellular signaling and, as a
result, cell fate. The rate constants of FGF2 binding to purified or cell surface HSPGs and FGFRs have been characterized (25). The rate constants of FGFR1 and FGF2 binding to heparin have also been investigated using optical biosensor methodology (34, 35). However, the kinetics of FGFR binding to heparin or HS have not been determined. In contrast, several studies have investigated the affinity of the interaction between FGFRs and heparins (29, 36-38). The values reported cover a very wide range from 10 nM to 104 µM. An explanation for this may be the different heparin and protein preparations or, more likely, the different techniques used. Furthermore, some of the studies differ in the presence of divalent cations, which have been observed to be required for the interaction of FGFR1 with heparin in one study (38).

Signaling by FGFRs has been shown to be regulated by specific HS saccharides in both a receptor and ligand-specific manner (15, 16). The ability of HS saccharides to determine which of FGFR1 IIC and FGFR2 IIC is activated by a common ligand suggests that different receptors have the capacity to bind differentially to HS sequences and that these differences may regulate FGF signaling (36). To investigate whether FGFRs interact differently with heparin/HS, we have compared the direct interaction of soluble FGFR1 IIC and FGFR2 IIC ectodomains with both heparin and porcine mucosal HS (PMHS) in the absence of FGFs. The results show that under the same conditions FGFR1 and FGFR2 bind both heparin and PMHS differently despite having the same general requirements for 6-O- and N-sulfation of glucosamine and 2-O-sulfation of IdUA. These receptors also bind heparin chains with different kinetics; FGFR2 possesses a faster association rate constant, resulting in a higher intrinsic affinity. These findings suggest that the dynamics of FGFR-HS binary complex formation are an important aspect of the mechanism by which HS/FGF interactions regulate FGF signaling.

**EXPERIMENTAL PROCEDURES**

**Materials**—PMHS was a gift from Organon (Oss, Netherlands). Bovine lung heparin and porcine intestinal mucosal heparin were purchased from Sigma. Chemically modified heparins were prepared from bovine lung heparin as previously described (39), NMR characterization of the modified heparins indicated that selective removal of 2-O- and N-sulfates had been 80% and 100% successful, respectively, with no other alterations, whereas 6-O-desulfation had removed 90% of 6-O-sulfates but also 30% of 2-O-sulfates.

**Production of Soluble FGFR Ectodomains**—Production of murine FGFR1 IIC and human FGFR2 IIC ectodomains as fusion proteins with human IgG1 Fc (FGFR-Fc) was as previously described (40), except that the receptors were separated from co-purifying HSPG material by washing with 0.5 M NaCl while immobilized on protein A-Sepharose (Amersham Biosciences). FGFR-Fc proteins were also expressed in the presence of [3H]glucosamine (PerkinElmer Life Sciences) (5 µCi/ml), which was added before transient transfection and maintained throughout expression. IgG1 Fc was purified by cleavage of the Fc using the human rhinovirus 3C protease, as previously described (40).

**Inhibition of FGF2/Heparin-mediated BaF3 Cell Mitogenesis by FGFR-Fc Proteins**—BaF3 cells transfected with FGFR1 IIC were maintained in RPMI 1640 growth medium supplemented with 10% fetal calf serum, 2 mM l-glutamine, 100 µunits/ml penicillin G, 50 µg/ml streptomycin sulfate, and 2 µg/ml interleukin 3 (R & D Systems). Dilutions of purified FGFR-Fc proteins in RPMI 1640 containing bovine lung heparin were added in RPMI growth medium. Cells were added to a final density of 10^4 cells/ml in medium without interleukin 3 and incubated at 37 °C in 5% CO2 for 72 h before determining cell viability using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) as described previously (41). Briefly, 10 µl of MTT (5 mg/ml in PBS) was added for 4 h at 37 °C. After clarification by the addition of 100 µl of 0.1 M SDS, 0.01 M HCl for 16 h, A_{570} values were measured using a microtiter plate reader ( Molecular Devices). As a positive control, the cells were incubated as above in growth medium containing 1 ng/ml interleukin 3, whereas negative controls used growth medium alone.

**Heparin-Sepharose Chromatography—**Radiolabeled FGFR-Fc or IgG1 Fc proteins (~5000 or 250 cpm/mmol, respectively) were applied to a 1 ml heparin-Sepharose HiTrap column (Amersham Biosciences) previously equilibrated in PBS using an HPLC system (Shimadzu). The column was washed with 10 column volumes of PBS, and bound protein was eluted with a linear gradient of NaCl in PBS (0–1 M over 30 min at 1 ml/min). 0.5-ml fractions were collected and analyzed for radioactivity by scintillation counting.

**Enzyme-Linked Immunosorbent Assay—**Three µg/ml streptavidin (ICN) in 0.1 M Na2CO3.HCO3 (pH 9.6) was incubated for 16 h at 4 °C in Maxisorp 96-well microtiter plates (Nunc). The plates were blocked with 1% (w/v) BSA (Sigma) in PBS for 2 h at room temperature, washed with PBS, 0.05% (v/v) Tween 20 (Sigma) (PBSST), and then incubated with biotinylated bovine lung heparin or PMHS (~5 or 75 µg/ml, respectively) in PBSST for 1–2 h at room temperature. The plates were washed with PBSST, and serial dilutions of FGFR-Fc proteins in 1% (w/v) BSA, PBSST were transferred to the wells and incubated at 4 °C for 16 h for binding to reach equilibrium. Nonspecific binding was determined by the addition of heparin or PMHS at 100× each FGFR concentration. The plates were washed in PBSST and incubated with goat anti-human IgG Fc horseradish peroxidase (Pierce) diluted 1:5000 in 1% (w/v) BSA, PBSST for 1 h at room temperature. After washing in PBSST the plates were developed with o-phenylenediamine (Dako), according to the manufacturer's instructions, and A_{490} values were measured using the microtiter plate reader. For competition experiments, the procedure was as detailed above, except that a constant concentration of FGFR-Fc was incubated with variable concentrations of soluble competitors in 1% (w/v) BSA, PBSST. Equal masses of competitors were used, since the polydispersity nature of GAGs renders molecular weight determinations of full-length chain mixtures, and therefore calculated molarities, inaccurate. Four-parameter logistic equations were fitted to data, plotted on a semi-logarithmic scale using non-linear regression with Prism software (Graphpad, UK).

**IAsys Biosensor Binding Studies—**Binding reactions were carried out in an IAsys resonant mirror biosensor at 20 °C using planar aminosilane surfaces derivatized with streptavidin according to the manufacturer's instructions (Thermo Labsystems, Cambridge, UK). Immobilization of biotinylated porcine mucosal heparin (ligand) on streptavidin derivatized surfaces and the binding of soluble FGFR-Fc to the heparin surface were as described for FGFs previously (13). Briefly, ligate (FGFR-Fc) was added to a known concentration in 30 µl of PBS, 0.02% (v/v) Tween 20 (PBS2T) at 37 °C for 2 h. After washing in PBSST the cuvette was washed 3 times with 50 µl of PBS2T, and the dissociation of bound ligate into the bulk PBS2T was followed over time. To remove residual bound ligate and to regenerate the immobilized ligand, the cuvette was washed three times with 50 µl of 2 M NaCl, 10 µM Na2HPO4 (pH 7.2). After a single wash with 50 µl of PBS2T, the dissociation of bound FGF-Fc into bulk PBS2T containing excess...
(5 mg/ml) heparin was also monitored. Binding parameters were calculated from the association and dissociation phases of the binding reactions using non-linear curve-fitting FastFit software (Affinity Sensors) provided with the instrument.

Several experimental precautions were taken to minimize the influence of artifacts due to mass transport, steric hindrance, ligate bivalency, or ligand heterogeneity (45–47). The amount of ligand immobilized was minimal, association curves were obtained using the minimal concentrations of ligate required to obtain an adequate response, whereas the dissociation of ligate from the surface was followed for high concentrations of ligate (greater than 700 nM). The FGFR-Fc proteins used in this study possess the Fc and hinge region of the human IgG1 variant and, hence, are purified as disulfide-linked dimers. Similar FGFR dimers have been found to bind FGF7 differently to analogous monomeric FGFRs (48), where the short hinge and single interchain disulfide bond, five residues from the hinge terminus nearest the Fc (51), provide the FGFR ectodomains of the FGFR-Fc molecules with limited flexibility but do not allow them to become juxtaposed. This combined with the precautions adopted makes it likely that the interactions characterized are those of FGFR ectodomains interacting with heparin chains without avidity effects.

RESULTS

Purification of FGFR Ectodomains as IgG1 Fc Fusion Proteins—FGFR1 and FGFR2 IIIc ectodomains, comprising Ig-like domains I-III, were expressed as soluble FGFR-Fc fusion proteins using human epithelial kidney (HEK) 293T cells. Analysis of purified FGFR-Fc proteins (washed with 0.5 M NaCl: see next section below) using SDS-PAGE (Fig. 1) showed single species of ~100 kDa. Glycosylation of the ectodomains and Fc would account for this apparent molecular mass being greater than the molecular mass of the fusion proteins predicted from the amino acid sequence (~69 kDa) and the diffuse nature of the bands. Western blot analysis confirmed these species contain human Fc (data not shown).

Endogenous HSPG Co-purifies with FGFR2-Fc—The use of a mammalian cell line for expression of the FGFR-Fc proteins prompted us to investigate whether endogenous HS, shed from the surface of the cells, was able to co-purify with the receptors. Preliminary experiments indicated that such material could block binding of the recombinant receptors to heparin (data not shown). Fusion proteins were expressed in the presence of [3H]glucosamine and purified on protein A-Sepharose as described under “Experimental Procedures.” The 0.5 M NaCl wash of FGFR2-Fc immobilized on protein A-Sepharose during purification was desalted and analyzed by Sepharose CL-6B gel filtration, untreated (A) or after sequential cleavage with alkali (B) and then nitrous acid (C) as described under “Experimental Procedures.” V₀, void volume; Vₜ, total volume.

Control experiments indicated that interleukin 3-stimulated proliferation was not affected at high concentrations of the soluble FGFR-Fc proteins (those that demonstrated maximal inhibition of FGFR2/heparin-dependent BaF3-R1 proliferation), suggesting that the soluble FGFR-Fc proteins are not toxic to the BaF3-R1 cells (data not shown). Furthermore, at concentrations of soluble FGFR-Fc proteins, which produced 90% inhibition of the response of BaF3-R1 cells to FGF2/heparin, increasing the concentration of FGF2 and heparin each by 8-fold restored the response of the cells to that observed in the absence of soluble FGFR-Fc (Fig. 3B). This indicates that the type (52), the material chromatographed predominantly in the total volume (Vₜ) of the column (Fig. 2C). Overall, the data indicate that the co-purifying material was an HSPG of ~120 kDa displaying HS chains with a mass of ~20 kDa.

FGFR2-Fc Is a More Potent Inhibitor of BaF3-R1 Cell Proliferation Than FGFR1-Fc—FGFR1-Fc and FGFR2-Fc, depleted of endogenous HS using a NaCl wash step as described above, were tested for their ability to inhibit FGF2/heparin-stimulated proliferation of an HS-deficient mouse lymphoblastoid cell line transfected with FGFR1 (BaF3-R1 cells). Inhibition was found to be dose-responsive for both FGFR1-Fc and FGFR2-Fc, with IC₅₀ values of 470 and 150 pM, respectively (Fig. 3A).

Fig. 1. Reducing SDS-PAGE analysis of purified FGFR-Fc proteins. FGFR-Fc fusion proteins were expressed using human epithelial kidney 293T cells and purified on protein A-Sepharose as described under “Experimental Procedures.” The proteins (~1.5 µg) were analyzed using 7.5% Tris-glycine discontinuous SDS-PAGE and staining with Gelcode blue stain reagent. Lane 1, protein standards; lane 2, FGFR1-Fc; lane 3, FGFR2-Fc.

Fig. 2. An HSPG from HEK 293T cells co-purifies with FGFR2-Fc during overexpression. FGFR2-Fc was expressed in the presence of [3H]glucosamine and purified on protein A-Sepharose as described under “Experimental Procedures.” The 0.5 M NaCl wash of FGFR2-Fc immobilized on protein A-Sepharose during purification was desalted and analyzed by Sepharose CL-6B gel filtration, untreated (A) or after sequential cleavage with alkali (B) and then nitrous acid (C) as described under “Experimental Procedures.” V₀, void volume; Vₜ, total volume.
The effect of the soluble FGFR-Fc proteins was due to specific interference with the response to FGF2. These experiments indicate that the recombinant FGFR-Fc proteins are, as expected, specific and potent inhibitors of FGF2/heparin-mediated proliferation as a consequence of competing with the cellular receptors for FGF2 and/or heparin. Importantly, in this assay FGFR2-Fc was a more potent competitor of endogenous FGFR1 signaling than FGFR1-Fc itself (Fig. 3A).

FGFR1-Fc and FGFR2-Fc Display Different Apparent Affinities for Heparin or PMHS — The increased potency of FGFR2-Fc as an inhibitor of FGF2/heparin-mediated cell proliferation with respect to FGFR1-Fc may reflect a difference in the interaction of the FGFRs with FGF2 or heparin. To compare the abilities of both proteins to bind heparin, FGFR1-Fc and FGFR2-Fc were bound to a heparin-Sepharose column and subjected to a linear gradient of NaCl. Both FGFR1-Fc and FGFR2-Fc interacted with heparin-Sepharose at physiological ionic strength and required 0.33 or 0.43 M NaCl, respectively, for elution (Fig. 4A). IgG1 Fc, produced by proteolytic cleavage of the FGFR-Fc fusion protein, did not interact with heparin-Sepharose at physiological ionic strength (data not shown). The elution of FGFR2-Fc at a higher concentration of NaCl than FGFR1-Fc suggests an interaction of higher apparent affinity.

The difference in the binding of the receptors to heparin was shown to occur over a range of FGFR-Fc concentrations using an enzyme-linked immunosorbent assay (Fig. 4B). The semi-logarithmic graphs also demonstrate that the binding is saturable. The EC50 values for FGFR1-Fc and FGFR2-Fc binding were 230 and 18 nM, respectively, indicating a higher apparent affinity of FGFR2 for heparin, in agreement with the heparin-Sepharose NaCl elution data.
Previous studies demonstrate that distinct sequences within PMHS chains differentially activate FGF2 mitogenesis via FGFR1 and FGFR2, suggesting that HS sequences may interact differently with receptors and as a result determine which receptor the ligand can engage (15). We therefore investigated the binding of FGFR1-Fc and FGFR2-Fc to PMHS chains. FGFR1-Fc and FGFR2-Fc bound immobilized PMHS over a series of FGFR-Fc concentrations, confirming that the receptors also interact with PMHS (Fig. 4C). The interaction of FGFR2-Fc with PMHS approaches saturation. The saturability of FGFR1-Fc binding was not investigated because of the prohibitively high concentration of receptor required. Accurate EC$_{50}$ values could not therefore be determined. However, the results do indicate that, as observed for heparin, PMHS interacts at a higher apparent affinity with FGFR2-Fc than FGFR1-Fc.

To confirm the specificity of the interactions of FGFR1-Fc and FGFR2-Fc with heparin and PMHS, the abilities of different GAGs to compete with the interactions between the receptors and immobilized heparin or PMHS were determined. Fig. 5, A–B, shows that PMHS is a less potent competitor than heparin of the interactions with immobilized heparin by at least 4 orders of magnitude. This may be due to PMHS containing a lower abundance of high affinity binding sequences for FGFR1-Fc and FGFR2-Fc than heparin.

Chondroitin sulfate was found to be a less potent competitor of the interactions than heparin by approximately 6 orders of magnitude (Fig. 5, A–B). In addition, chemically over-sulfated heparin was a marginally less effective competitor than heparin (Fig. 5, A–B). Hence, there is no simple relationship between the number of negative charges per disaccharide unit and the ability of different GAGs to compete with heparin for binding to FGFR1-Fc and FGFR2-Fc. Similar results were obtained for the abilities of soluble GAGs to inhibit the binding of FGFR1-Fc and FGFR2-Fc to immobilized PMHS. Soluble PMHS was found to be a more potent competitor than chondroitin sulfate, whereas over-sulfated heparin was of similar or lower potency to heparin (Fig. 5, C–F). Overall these data indicate that the interactions are mediated by specific sequences within the heparin and PMHS chains rather than by nonspecific electrostatic interactions.

The Interactions of FGFR1-Fc and FGFR2-Fc with Heparin Involves the Same Sulfate Groups—Comparison of the interactions of heparin saccharides with FGFR1 and FGFR2 in the ternary complex crystal structures (53) indicated that in the presence of an FGF, FGFR1 interacted with an additional type
FGFR1 and -2 Interact Differently with Heparin/HS

sulfate selectively removed from heparin differentially affects its ability to interact with FGFR1-Fc and FGFR2-Fc. This supports the data above indicating that specific sequences mediate the interactions but again suggests that the interactions of both receptors involve the same types of sulfate groups.

**FGFR1-Fc and FGFR2-Fc Display Different Interaction Kinetics with Heparin**—To determine whether the dynamics of the interactions also differed, the kinetics of FGFR1-Fc and FGFR2-Fc binding to heparin were determined using an optical biosensor. Association rate constants ($k_a$) were calculated using binding curves for minimal concentrations of FGFR-Fc, which obeyed a one-site binding model (Fig. 7). The values for $k_a$ calculated using alternative analytical methods (Table I) are equivalent and within combined fractional errors. The binding of FGFR1-Fc and FGFR2-Fc to heparin exhibited mean $k_a$ values of $1.9 \times 10^6$ and $2.1 \times 10^6$ M$^{-1}$s$^{-1}$, respectively (Table I). FGFR2-Fc therefore displays a 10-fold faster $k_a$ than FGFR1-Fc.

Both FGFR-Fc proteins had mean dissociation rate constants ($k_d$) of $1 \times 10^{-2}$ s$^{-1}$ (Table I). Because of its faster $k_a$, the dissociation of FGFR2-Fc from heparin was also measured independently in the presence of excess soluble heparin to assess the possibility of re-binding artifacts reducing the dissociation rate constant. Inclusion of excess heparin in the dissociation buffer only increased $k_d$ approximately 2-fold for FGFR2-Fc, rendering it ~2-times faster than for FGFR1-Fc (Table I).

The mean equilibrium dissociation constants ($K_d$) for the interaction of FGFR1-Fc and FGFR2-Fc with immobilized heparin were calculated from the kinetic rate constants as 63 and 13 nM, respectively (Table I). Independent calculation of these values from a graph of extent of binding against concentration of FGFR-Fc (data not shown) produced similar values for FGFR2-Fc but not FGFR1-Fc (Table I). This latter method of calculating $K_d$ used higher concentrations of FGFR1-Fc (72–580 nM), which showed a susceptibility to obey kinetics described by a two-site model. The affinity of the interaction of FGFR1-Fc calculated from the kinetic parameters is, therefore, considered to more accurately reflect the intrinsic affinity of this interaction. Unlike for heparin, the binding of the proteins to PMHS could not be described by a one-site binding model even at minimal concentrations of FGFR-Fc. This is probably due to the more extensive heterogeneity of the structure of PMHS chains with respect to those of heparin. As a result the kinetics of receptors binding to PMHS could not be accurately determined.

**DISCUSSION**

The fact that HS is required to stimulate sustained signaling and mitogenesis by FGFR2 (33) suggests that the relationship between the dynamics of complex assembly and intracellular signaling may underlie the role of HS as a co-receptor. The ability of distinct saccharides from HS to differentially regulate the activation of FGFR1 and FGFR2 by a common FGF ligand suggests that HS may interact differently with different FGFRs and by so doing regulate the ability of the ligand to stimulate cell proliferation (15). Comparison of the interactions of different receptors with HS in the absence of FGF as described here, especially dynamic properties such as kinetics, provides important new information regarding the mechanism of regulation of FGF signaling by HS.

We have characterized the specific interactions of FGFR1-Fc and FGFR2-Fc with heparin and PMHS at physiological ionic strength and pH. This is the first time that FGFR2 has been shown to interact with an HS species other than heparin. The interactions of FGFR1 and FGFR2 with heparin both involve the same types of sulfate groups: 6-O-, 2-O-, and N-sulfates. In
In contrast, a comparison of the crystal structures of the receptors as ternary complexes with FGFs and heparin saccharides (53) suggests differences in the sulfates involved in the interaction of heparin with the two receptors; FGFR1 binds all three sulfate types, and FGFR2 binds just 6-O and N-sulfates. This disparity between studies may reflect differences in the binary and ternary complexes or, alternatively, between interactions occurring in solution and in crystals.

The data of this study also show that, despite an overall requirement for the same sulfate groups, the interactions of the two receptors with heparin exhibit markedly different association rate constants and hence affinities. The apparent affini-
FGFR1 and -2Interact Differently with Heparin/HS

**FIG. 8.** Kinetic regulation model for activation of FGF signaling. A ternary complex of 2 FGF:2 FGFR:1 HS chain is formed by the three components, each interacting with each other. Multiple factors affect the kinetics of the interactions involved in ternary complex formation and, hence, affect the kinetics of intracellular signal transduction pathways, which in turn determine the outcome of signaling. I, II, III; immunoglobulin-like domains of FGFRs; PM, plasma membrane; TK, tyrosine kinase domains of FGFRs.

**TABLE I**

|                | $k_a$ | $r^b$ | $k_f$ | $r^d$ | $k_a$ | $K_a$ (equilibrium)$^e$ | $K_f$ (kinetic)$^e$ |
|----------------|-------|-------|-------|-------|-------|--------------------------|---------------------|
| $1 / h_{max}$ | $h_{max}$ | $1 / h_{max}$ | $r_{max}$ | $r_{max}$ | $h_{max}$ | $K_a$ (equilibrium)$^e$ | $K_f$ (kinetic)$^e$ |
| FGFR1-Fc       | 1.9 ± 0.3 × 10^5 | 0.98 | 2.0 ± 1.1 × 10^5 | 0.96 | 1.2 ± 0.2 × 10^{-2} | ND | 450 ± 400 | 63 ± 17 |
| FGFR2-Fc       | 2.1 ± 0.6 × 10^6 | 0.97 | 1.5 ± 0.7 × 10^6 | 0.95 | 1.3 ± 0.1 × 10^{-2} | 2.7 ± 0.1 × 10^{-2} | 10 ± 6 | 13 ± 4 |

$^a$ $k_a$ is derived from linear regression of a graph of $k_a$ against the concentration of FGFR-Fc and is the mean of three independent experiments ± the combined S.E.

$^b$ $r$ is the correlation coefficient of the linear regression through the values for $r$ against the concentration of FGFR-Fc.

$^c$ $k_f$ is derived from the ratio of slope/$h_{max}$, calculated using the means of values, each calculated from three independent experiments ± the combined S.E. The slope is the gradient of a graph of initial rate against the concentrations of FGFR-Fc. $B_{max}$ is the maximum extent of binding, calculated from non-linear regression (using a one-site model) of a graph of the extent of binding against the concentrations of FGFR-Fc, plotted using the extents calculated by non-linear curve-fitting of a one-site model to the association phase.

$^d$ $r$ is the correlation coefficient of the linear regression through the values for initial rate against concentrations of FGFR-Fc.

$^e$ $K_a$ (equilibrium) is derived from non-linear regression (using a one-site model) of a graph of the extent of binding against the concentrations of FGFR-Fc, plotted using the extents calculated by non-linear curve-fitting of a one-site model to the association phase. It is the average of three independent experiments ± the combined S.E.

$^f$ $K_f$ (kinetic) is calculated from the ratio of $k_f / k_a$ for FGFR1-Fc and $k_f / k_a$ for FGFR2-Fc. S.E. is the combined S.E. of the two parameters.

FGFRs ↔ FGFs

HS-related factors affecting complex formation:

- Interaction of active HS sequence with FGFs & FGFRs (rate constants/abundance/conformations)
- Context of active HS sequence in chain (conformational restraints/competing binding sites)
- HS location (dimensionality/proximity to FGFRs)
- Competing HS-binding proteins (rate constants/abundance)

but exhibits subtle differences in sequence (8). Comparison of the K18K sequence of the FGFR1 and FGFR2 used in these studies indicates four amino acid differences. It is possible, therefore, that FGFR1 and FGFR2 bind sequences within heparin chains that are in different conformations and that this difference in binding is reflected by the 10-fold difference in association rate constant that we observed. Because evidence suggests that many protein-binding sites are located in the NS domains of HS chains (6), the difference in apparent affinity of FGFR1 and FGFR2 binding to PMHS may also reflect binding to sequences of different conformation. Furthermore, the kinetics of binding of FGFRs to sequences within heparin chains may to some extent reflect the kinetics of binding to highly sulfated sequences within NS domains.

Given the kinetic measurements made in the present work, we propose a model where the kinetics of the binding of both FGFs and FGFRs to sequences within HS chains determine the

eties of the receptors for PMHS were also different. Heparin and the NS domains of HS possess a high abundance of IdoUA 2-sulfate, a residue which has been shown to adopt different conformations (54) and, therefore, may bestow upon the chains a degree of flexibility absent from unmodified regions (55). Sulfation elsewhere has also been shown to affect the prevalence of particular IdoUA 2-sulfate conformations (54) and glycosidic linkage geometry (56). Consequently, heparin and the NS domains of HS are likely to contain flexible sequences whose conformational properties are dependent upon the pattern of sulfation. In the process of protein binding, alterations in the conformation of the binding sequences within heparin and the NS domains of HS may occur to enhance complementarity with different protein-binding sites. The major region of FGFR1 that interacts with heparin was shown to contain a stretch of amino acids rich in positively charged residues (K18K sequence) (28). This region is conserved across FGFRs...
frequency at which the active ternary complex is formed and, hence, regulate the dynamics of FGF-dependent signaling (Fig. 8). We also suggest that the conformation of the protein-binding sites within NS domains may be influenced by the pattern of idurionate 2-sulfate residues and sulfate groups of adjacent residues as well as by adjacent NA domains. The abundance of the NA domains may also influence the flexibility of the whole chain. By affecting the kinetics of protein binding and ternary complex formation, these structural features may, therefore, be crucial factors in the proposed kinetic regulation model.

Such a model may explain the ability of specific HS saccharides to differentially regulate the ability of FGF2 to activate cell proliferation via different FGFRs and the unanticipated ability of a decasaccharide exhibiting a low level of sulfation to be a more potent activator than heparin of FGF2 signaling through FGFR2 (15). Furthermore, the probable lack of flexibility of NA domains may explain why HSPGs and HS chains that are unable to activate FGF2 signaling (and can even act as inhibitors) are converted into activators on cleavage of the chains, within these domains by heparitinase I (13, 57, 58).

In vivo, the dynamic and tightly regulated nature of HS chain biosynthesis may enable cells to alter which sequences are expressed and, as a result, the kinetics of complex formation. This would enable cells to fine tune their spatial and temporal responses to FGF ligands whose expression is overlapping.

Other factors would also be of importance in vivo in the context of such a model for the mechanism by which HS regulates FGF signaling. Multiple proteins are known to bind HS, and therefore, individual HS chains are likely to act as “multireceptors” for different proteins (59). The binding of other proteins may, therefore, also regulate FGF signaling by masking binding sites for FGFs and FGFRs on cellular HS chains (60). Several proteins have been measured to exhibit similar association rate constants to FGFs and FGFRs for binding to heparin or HS chains (10^{-4}–10^{6} M^{-1} s^{-1}). The dynamics of ternary complex formation and, consequently, the activation of FGF-signaling pathways may, therefore, be determined by the relative rates of association and abundance of competing proteins binding to FGF/FGFR binding sequences within the HS chains. Different FGFs and FGFRs may also be in competition for binding sites; hence, their relative abundance and rate constants for a particular sequence may dictate the type of ternary complex formed. The 10-fold greater association rate constant of FGFR2 with respect to FGFR1 for binding heparin suggests that this receptor would have a competitive advantage for binding heparin-like sequences. Furthermore, in order for an active HS-FGF-FGFR ternary signaling complex to be formed, the FGF and FGFR binding sequences within HS are likely to be in close spatial proximity, enabling FGFs and FGFRs to interact with both HS and each other. Indeed, this is indicated by the crystal structures of the ternary complexes of FGFs, FGFRs, and heparin oligosaccharides (61, 62). The above factors may, therefore, influence the probability of FGFRs and FGFs finding adjacent sites available and, hence, the formation of active ternary complexes.

The affinities of the interaction of the FGFs with heparin are in the 10^{-8}–10^{-6} M range and similar to those of FGF1 and FGF2 for heparin (34, 35). It has been observed that typical concentrations of HSPGs on the cell surface are in the range 10^{5}–10^{6} molecules/cell (57, 63). Several studies calculate that there are 0.5–5 × 10^{4} FGFR molecules/cell (64). Hence, there is probably a large excess of HSPG with respect to FGF on the cell surface. Furthermore, the effective concentrations of FGFs and HSs are likely to be increased by crowding effects (65) and their immobilization in cell membranes, which reduces the dimensionality of diffusion. The high intrinsic affinities that we have observed for the interactions between FGFs and heparin coupled with the excess of HSPG on cell surfaces suggest that if a significant proportion of HS chains on cells contain appropriate binding sequences, then the FGFs will be saturated with HS. This is of notable significance to the mechanism of FGF-ligand engagement, since as a consequence, extracellular FGFs are only likely to encounter FGFs as complexes with HS. The similar innate affinities of FGFs with respect to FGFRs for heparin sequences may make a similar argument apply to FGFs. This would depend on the relative effective concentrations of FGFs and HSPGs and the degree of crowding in the extracellular matrix. Ternary complex assembly may, therefore, occur by HS acting as a “catalyst of molecular encounter” under restricted dimensions of diffusion (66).

In summary, we have demonstrated specific interactions between FGFRs and bioactive HS/heparin that exhibit markedly different kinetics for individual FGFRs. We propose a mechanistic model where the kinetic parameters of the HS/FGFR interaction are important in determining the output of FGF signaling and, hence, cell fate. Future studies to determine the sequence of saccharides that differentially activate FGF2 signaling via different receptors and the kinetics of their interactions with FGFRs, FGFs, and other prominent extracellular matrix proteins as well as their abilities to stimulate transient or sustained cell signaling will provide important information regarding this kinetic regulation model.

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REFERENCES

1. Rapraeger, A. C., Krufka, A., and Olwin, B. B. (1991) Science 252, 1705–1708
2. Yuwono, A., Klagsbrun, M., Etkin, J. D., Leder, P., and Ornitz, D. M. (1991) Cell 64, 841–848
3. Fernig, D. G., and Gallagher, J. T. (1994) Prog. Growth Factor Res. 5, 355–377
4. Stavridis, M., Fraser, J. M., McDonald, M., Yuan, S., White, D., Grandison, P., Kumble, K., Watson, J. D., and Murison, J. G. (2001) Gene 271, 171–182
5. Bernfield, M., Gotti, M., Park, P. W., Rezes, O., Fitzgerald, M. L., Lincecum, J., and Zako, M. (1999) Annu. Rev. Biochem. 68, 729–777
6. Lindahl, U., Kusche-Gullberg, M., and Kjellén, L. (1998) J. Biol. Chem. 273, 24979–24982
7. Stringer, S. E., Mayer-Proeschel, M., Kalyani, A., Ran, M., and Gallagher, J. T. (1999) J. Biol. Chem. 274, 25455–25460
8. Conrad, H. E. (1998) Heparin Binding Proteins, Academic Press, Inc., New York
9. Kreuger, J., Salmivirta, M., Sturiale, L., Gimenez-Gallego, G., and Lindahl, U. (2001) J. Biol. Chem. 276, 30744–30752
10. Ishihara, M. (1994) Glycobiology 4, 817–824
11. Guimond, S., Macaronna, M., Olwin, B. B., Lindahl, U., and Rapraeger, A. C. (1993) J. Biol. Chem. 268, 12705–12711
12. Ornitz, D. M., Xu, J., Calvin, J. S., McEwen, D. G., MacArthur, C. A., Coulier, F., Gao, G., and Goldfarb, M. J. (1996) J. Biol. Chem. 271, 15292–15297
13. Rahmouni, H., Chen, H. L., Gallagher, J. T., Rudland, P. S., and Fernig, D. G. (1998) J. Biol. Chem. 273, 7303–7310
14. Kan, M., Wu, X., Wang, F., and McKeenan, W. L. (1999) J. Biol. Chem. 274, 15947–15952
15. Guimond, S. E., and Turnbull, J. E. (1999) Curr. Biol. 9, 1343–1346
16. Pye, D. A., Vives, R. R., Hyde, P., and Gallagher, J. T. (2000) Glycobiology 10, 1183–1192
17. Ostonovsky, O., Berman, B., Gallagher, J., Mulloy, B., Fernig, D. G., Delledebbe, M., and Ron, D. (2002) J. Biol. Chem. 277, 2444–2453
18. Chang, Z., Meyer, K., Rapraeger, A. C., and Friedl, A. (2000) FASEB J. 14, 137–144
19. Allen, B. L., Filla, M. S., and Rapraeger, A. C. (2001) J. Cell Biol. 155, 845–858
20. Uematsu, F., Kan, M., Wang, F., Jiang, J. H., Luo, Y., and McKeenan, W. L. (2000) Biochem. Biophys. Res. Commun. 273, 830–836
21. Schlessinger, J., Lax, I., and Lennarz, W. J. (1993) Cell 73, 357–360
22. Faham, S., Hileman, R. E., Fromm, J. R., Linhardt, R. J., and Rees, D. C. (1996) Science 271, 1116–1120
23. Spivak-Kroizman, T., Lobo, M. A., Dikix, I., Ladbury, J. E., Panicotti, D., Huang, J., Jaye, M., Crumley, G., Schlessinger, J., and Lax, I. (1994) Cell 79, 1015–1024
24. Kwan, C. P., Venkataraman, G., Shriver, Z., Raman, R., Liu, D., Qi, Y., Varticovski, L., and Sassekharan, R. (2001) J. Biol. Chem. 276, 23421–23429
25. Nugent, M. A., and Edelman, E. R. (1992) Biochemistry 31, 8876–8883
26. Bogiani, M., Mansukhani, A., Dell’Era, P., Bellota, P., Basilo, C., Rifkin, D. B., and Moscatelli, D. (1994) J. Biol. Chem. 269, 3976–3984
27. Rusnati, M., Coltrini, D., Caccia, P., Dell’Era, P., Zoppetti, G., Orreste, P., Valassina, B., and Presta, M. (1994) Biochem. Biophys. Res. Commun. 203,
