Heparin Is Essential for a Single Keratinocyte Growth Factor Molecule To Bind and Form a Complex with Two Molecules of the Extracellular Domain of Its Receptor

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ABSTRACT: Keratinocyte growth factor (KGF or FGF-7) is a member of the heparin binding fibroblast growth factor (FGF) family and is a paracrine mediator of proliferation and differentiation of a wide variety of epithelial cells. To examine the stoichiometry of complexes formed between KGF and its receptor, we have utilized a soluble variant of the extracellular region of the KGF receptor containing two tandem immunoglobulin-like loops, loops II and III (sKGFR). Ligand−receptor complexes were examined by size exclusion chromatography, light scattering, N-terminal protein sequencing, and sedimentation velocity. In the presence of low-molecular mass heparin (∼3 kDa), we demonstrate the formation of complexes containing two molecules of sKGFR and one molecule of KGF. In the absence of heparin, we were unable to detect any KGF−sKGFR complexes using the above techniques, and additional studies in which sedimentation equilibrium was used show that the binding is very weak ($K_d \geq 70$ μM). Furthermore, using heparin fragments of defined size, we demonstrate that a heparin octamer or decamer can promote formation of a 2:1 complex, while a hexamer does not. Utilizing the highly purified proteins and defined conditions described in this study, we find that heparin is obligatory for formation of a KGF−sKGFR complex. Finally, 32D cells, which appear to lack low-affinity FGF binding sites, were transfected with a KGFR−erythropoeitin receptor chimera and were found to require heparin to achieve maximal KGF stimulation. Our data are consistent with the previously described concept that cell- or matrix-associated heparan sulfate proteoglycans (HSPGs) and FGF ligands participate in a concerted mechanism that facilitates FGFR dimerization and signal transduction in vivo.

The FGFs[1] are a family of heparin binding growth factors which at present contains at least 17 members, including acidic FGF (aFGF, FGF-1), basic FGF (bFGF or FGF-2), KGF (FGF-7), FGF-8, FGF-9, KGF-2 (FGF-10), FGF-11, and the oncogene gene products int-2 (FGF-3), hst-1 (K-FGF or FGF-4), FGF-5, and hst-2 (FGF-6). The first member of this family identified, aFGF, although initially characterized as stimulatory with respect to fibroblast growth (*1*, *2*), has been subsequently shown to be the most promiscuous of the FGFs and to stimulate growth and differentiation of a wide variety of cells of both mesenchymal and epithelial origin. Indeed, most cell types have been found to be stimulated either directly or indirectly by at least one of the FGF family members. Keratinocyte growth factor (KGF) was originally isolated from conditioned media of human embryonic lung fibroblasts and was shown to be unique among FGF family members in its specificity toward cells of epithelial origin alone (*3*). More recently FGF-10, the FGF family member most like KGF, has also been shown to exhibit this epithelial cell specificity (*4*, *5*). KGF is expressed by stromal cells and stimulates the growth and differentiation of a variety of epithelial cells, acting as a paracrine mediator of mesenchymal−epithelial communication (reviewed in ref *6*).

The mechanisms by which FGFs bind and elicit a biological response from target cells are still relatively obscure and somewhat controversial. Using radiolabeled FGF ligands, two classes of receptors on the cell surface have been detected and are referred to as "low-affinity" and "high-

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[1] Abbreviations: FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; KGF, keratinocyte growth factor; sKGFR, soluble keratinocyte growth factor receptor; EPO, erythropoeitin; EPOR, erythropoeitin receptor; HS, heparan sulfate; HSPG, heparan sulfate proteoglycans; GAG, glycosaminoglycan; PBS, Dulbecco's phosphate-buffered saline (without calcium or magnesium); SEC, size exclusion chromatography.

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affinity" binding sites. The low-affinity binding sites were identified as heparan sulfate proteoglycans (HSPG) present in large numbers on the cell surface and in the extracellular matrix (*7*, *8*). The high-affinity binding sites for FGFs have been shown to be one of the four identified FGFRs to date, FGFR1−FGFR4 (*9*−*11*). These receptors contain an intracellular tyrosine kinase domain, a transmembrane region, and an extracellular region containing two or three immunoglobulin (Ig)-like loops (for a review, see ref *11*). The amino-terminal Ig-like loop I has been shown to be dispensable, and receptor variants containing only the Ig-like loop II and the membrane proximal Ig-like loop III have been found to exhibit equivalent degrees of binding to the variants containing all three loops (*12*). The Ig-like loop II is highly conserved among the FGFRs and has been proposed to constitute the minimal binding site for all FGF polypeptides (*10*, *11*).

Alternative splicing of the extracellular domain of FGFRs confers unique and differential affinity for the FGF ligands. Acidic FGF binds to all known FGF receptors (FGFR1−FGFR4), including splice variants (*13*). Two alternative exons encode the carboxy-terminal half of Ig-like loop 3 and are designated exon IIIb and exon IIIc. KGF only binds well to the FGFR2(IIIb) splice variant, also known as the KGFR (*12*, *14*−*17*), whereas bFGF binding to this variant is poor (*12*, *16*, *17*). Both aFGF and bFGF have been shown to bind equally well to FGFR2 (FGFR2-IIIc) and FGFR1 (*18*). The epithelial cell-specific expression of the KGFR (FGFR2-IIIb) and the stromal cell-specific expression of KGF therefore appear to constitute a directionally specific system for communication between stromal and epithelial cells within tissues.

Heparan sulfate proteoglycans are a diverse group of proteins which contain at least one heparan sulfate chain attached to a polypeptide through a tetrasaccharide linkage (for a review, see refs *19* and *20*). Heparan sulfate is synthesized by most vertebrate cells (*21*), and HSPG sites are present on most cells known to naturally contain FGFRs. Several studies have sought to determine whether heparin or HSPG sites are required for the interaction of FGFs with their receptors. Heparin has been reported to be required for both cell-free and cell-surface binding of bFGF to FGFR1 and FGFR2 (*22*−*25*). On the basis of these results, models have been proposed (e.g., "an induced-fit model") in which bFGF must first bind to a HSPG site and then undergo a conformational change allowing it to recognize receptor (*9*, *20*, *22*). Yet similar studies of the influence of heparin and HSPGs on the binding of KGF to the KGFR have suggested that heparin is not required for formation of a ligand−receptor complex (*26*−*28*), and several reports have proposed that the presence of heparin or HSPG sites is actually inhibitory with respect to formation of the KGF−KGFR complex (*26*, *28*, *29*). In contrast to those reports, Jang et al. (*30*) have reported recently that HS or heparin is required for both high-affinity KGF binding to the KGFR and activation of KGFR on cells.

Although FGFR signal transduction has been thought for some time to require receptor dimerization (*31*), two models have surfaced to account for this process. In the first model, the signal-transducing complex has been proposed to contain two 1:1 FGF−FGFR complexes cross-bridged via HS or heparin, giving a 2:2 molar ratio of FGF to FGFR (*9*, *23*, *32*). In a second model, a 2:1 molar ratio of FGFR to FGF has been suggested to result in signal transduction (*33*). Pantoliano et al. (*33*) have performed an intensive study on the interactions of a soluble, purified FGFR1 (sFGFR1) with both low-molecular mass heparin and bFGF. They have found, in contrast to the induced-fit model described above, that bFGF forms a complex with sFGFR1 in the absence of heparin and that this complex has a 1:1 molar ratio. In the presence of heparin, they report that the affinity of sFGFR1 for bFGF was increased ∼10-fold and a 2:1 sFGFR1−bFGF complex was detected (*33*).

To investigate the stoichiometry of the KGF−KGFR interaction, and to further clarify the role of HS or heparin for this system, we have produced and purified to homogeneity a soluble KGFR (sKGFR) and explored its interaction with KGF by size exclusion chromatography, light scattering, and analytical ultracentrifugation. To our surprise, in the absence of heparin, we were unable to detect any strong KGF−sKGFR complexes. We show that under these conditions some very weak binding (K_d > 70 μM) for forming 1:1 complexes does occur. This heparin requirement for the interaction of the sKGFR with KGF seems to exceed that observed previously for bFGF and sFGFR1 (*33*), since we do not observe complexes at physiologically relevant concentrations in the absence of heparin. Upon addition of low-molecular mass heparin, we observe exclusively sKGFR−KGF complexes with a 2:1 stoichiometry. When using heparin fragments of different lengths, we see 2:1 complexes only for fragments longer than a hexamer. To explore the heparin requirement for KGF binding to KGFR on cells, we have transfected 32D myeloid cells that lack low-affinity FGF binding sites (e.g., HSPG) with a chimeric receptor which contains the extracellular region of the KGFR and the transmembrane and intracellular regions of the EPO receptor. This transfected cell line (32D-KECA) was found to require heparin to achieve maximal KGF stimulation. Heparin therefore appears, in the highly defined systems we have examined, to be essential for formation of a stable KGF complex with the KGF receptor.

EXPERIMENTAL PROCEDURES

Production and Purification of Proteins. For expression of the recombinant two-domain human KGF receptor (domains II and III), a DNA fragment encoding Ig-like loops II and III (Figure 1) of the extracellular region of the KGFR was subcloned between the *Bam*HI and *Hin*dIII sites of a pMelBac B baculovirus transfer vector, and the plasmid was cotransfected with Bac-N-Blue viral DNA (Invitrogen) into Sf9 insect cells. Recombinant virus stocks were amplified using Sf9 cells to yield a high-titer virus stock. sKGFR was secreted into the medium by High-5 cells infected with this virus stock. The cell culture medium was harvested 48−72 h postinfection. The recombinant human KGFR protein with extracellular Ig-like loops II and III was then purified from the conditioned medium using two-step chromatography. A half-liter of the harvested conditioned medium was concentrated and diluted 5-fold with water, and the pH was adjusted to 7.8 by adding 1 M Tris-HCl (pH 8.0). The sample then was loaded onto a SP-HiTrap column (5 mL, Pharmacia). The sKGFR was eluted by conducting a salt gradient; an equal volume of 1 M ammonium sulfate in PBS buffer was added, and further purification was achieved by hydrophobic

A.

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  1   MKFLVNVALVFMVVYISYIYADPMAPYWTNTEKMEKRLHAVPAANTVKFR--

        [------------------Ig-like Loop II--------------------]
 51 --CPAGGNPTPTMRWLKNGKEFKQEHRIGGYKVRNQHWSLIMESVVPSDKGNYTC--

104 --LVENEYGSINHTYHLDVVERSPHRPILQAGLPANASTVVGGDVEFV--

        [-----------------------Ig-like Loop III----------------------------]
150 --CKVYSDAQPHIQWIKHVEKNGSKYGPDGLPYLKVLKHSGINSSNAEVLALFNVTEADAGEYICC--

213 --KVSNYIGQANQSAWLTVLPKQQAPGREKEITASPDYLE
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B.

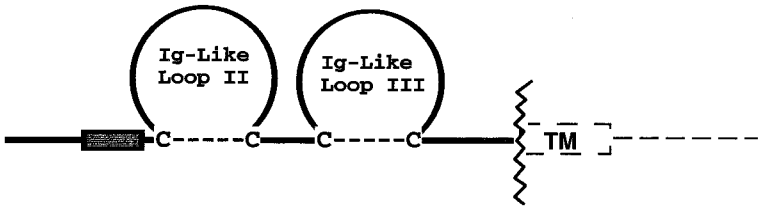

FIGURE 1: Structure of sKGFR. (A) Amino acid sequence of the soluble KGFR utilized in these studies and some notable features of the receptor. The amino terminus of the mature secreted protein was found by N-terminal sequencing to be the aspartic acid residue located at position 22. The melittin signal sequence is shown in italics and includes amino acid residues 1−21. Cysteine residues (in bold and double-underlined) that define Ig-like loops II and III are indicated, as well as the loops themselves. Amino acid residues of the receptor believed to be involved with heparin binding (*11*, *56*) are underlined. Exon IIIb of FGFR2 is indicated in bold. (B) A cartoon of the KGF receptor utilized in these studies. The extracellular region of a two-loop variant of the KGFR is shown to the left of the wavy vertical line. Carboxy-terminal residues DYLE of the soluble receptor described in part A immediately precede the transmembrane (TM) region of the cell-surface receptor. The region believed to be involved in heparin binding is indicated by a solid rectangular box.

chromatography on a phenyl-Sepharose column (1 mL, Pharmacia). The sKGFR was collected in the flow-through fraction, and diafiltered in PBS. An *Escherichia coli*-derived recombinant human KGF variant was prepared as described previously (*34*). Protein concentrations were measured spectrophotometrically using calculated molar extinction coefficients (*35*). An $E^{0.1\%}_{280}$ of 1.2 was used for KGF. For baculovirus-derived sKGFR, the $E^{0.1\%}_{280}$ (=1.65) and any mass/volume concentrations given are representative of the polypeptide portion only. Molecular mass values calculated from amino acid sequence alone were 16 700 Da for the KGF variant and 25 300 Da for sKGFR (polypeptide portion only, excluding attached carbohydrate).

Generation of Chimeric Receptor cDNA and Cell Transfection. The human keratinocyte growth factor receptor cDNA was cloned at Amgen and corresponds to GenBank accession number M80634. The murine EPOR cDNA sequence has been reported previously (*36*). DNA encoding the chimeric KGFR−EPOR was generated with a two-step polymerase chain reaction (PCR) (*37*) and contains the extracellular domain of the human KGFR [amino acids (aa) 1−1546] and the transmembrane and intracellular domains of the murine EPOR (aa 272−507). The resulting chimera is called KECA (K = KGFR, E = EPOR, C = chimera, and A = first in this series). Constructs were confirmed to be correct by DNA sequencing. Chimeric receptor DNA was subcloned into pLJ (*29*), a eukaryotic vector for transfection into 32D cells. Culture and transfection of 32D (clone 3) cells were carried out as previously described for other EPOR chimeras (*38*).

DNA Synthesis and Proliferation Assays. For the DNA synthesis assay, 32D vector control cells or pooled 32D KECA cells selected in KGF and all doses of heparin (grade I, porcine intestinal mucosa-derived, Sigma) were washed extensively with PBS and seeded into 96-well U-bottom plates at 10 000 cells/well in RPMI 1640/10% FBS. IL-3, various doses of KGF, or additional RPMI 1640/10% FBS was added, and the cells were incubated for 48 h. [^{3}H]-Thymidine (ICN, 1 μCi/mL) was added, and cells were labeled for 4 h. Cells were harvested, and the amount of incorporated (ethanol precipitable) radioactivity was quantified by scintillation counting in a Betaplate reader (Wallac). The 32D KECA cells were utilized for analysis of the heparin dosage-dependent proliferation detected by a Alamar Blue (AccuMed) fluorescence assay, which was similar to the method described previously (*39*). The intensity of fluorescence measured reflects the extent of cellular proliferation. Heparin was diluted to various concentrations with diluent containing KGF. The heparin−KGF solutions were added to 32D KECA cells in 96-well plates followed by incubation at 37 °C for 48 h. An amount of Alamar Blue equal to 20% of the culture volume was then added to the wells. Plates were incubated for an additional 24 h at 37 °C and then analyzed on a Cyto Fluor (PerSeptic Biosystems) fluorescence plate reader with an excitation wavelength of 530 nm and an emission wavelength of 590 nm.

Size Exclusion Chromatography. Size exclusion chromatography of KGF, sKGFR, and incubated mixtures of KGF, sKGFR, and heparin 3000 (Pharmacia; average molecular mass of 3000 Da) was carried out using a Superose 12 column (1 cm × 30 cm, Pharmacia) or a TSK G3000 XL (1 cm × 30 cm, TosoHaas) column equilibrated with or without 30 μg/mL heparin 3000 in 2× PBS (pH 7.4) (Gibco) at room temperature with a flow rate of 0.7 mL/min and attached to

a Hewlett-Packard 1050 liquid chromatography system. The detection wavelength was set at 215 nm.

N-Terminal Protein Sequencing Analysis. Proteins were sequenced using a Hewlett-Packard G1000A protein sequencer, equipped with a Hewlett-Packard 1050 liquid chromatography system and a Chem Station for on-line analysis of the PTH-amino acids.

Laser Light Scattering−Size Exclusion Chromatography. The on-line light scattering−chromatography system used three detectors connected in series: a Hewlett-Packard 1050 HPLC system (absorbance at 280 nm), a Wyatt Technologies Mini-Dawn laser light scattering detector, and a Hewlett-Packard refractive index detector (HP 1047A). A Superose 12 column (1 cm × 30 cm; Pharmacia) equilibrated with 2× PBS (pH 7.4) (Gibco) containing 30 μg/mL heparin 3000 was used at room temperature with a flow rate of 0.7 mL/min. Calibration was accomplished using ribonuclease, ovalbumin, and bovine serum albumin monomer and dimer (Sigma) as molecular mass standards.

Sedimentation Velocity. Sedimentation velocity experiments were carried out at 60 000 rpm and 20 °C in a Beckman Optima XL-A centrifuge, with concentrations monitored by absorbance scans at 230 nm. The resulting data were analyzed by the DCDT method (*40*). The defined heparin hexamer, octamer, and decamer oligosaccharides utilized in these studies were obtained from Celsus (Cincinnati, OH). According to the manufacturer, these materials are homogeneous in size, but heterogeneous in chemical structure.

Sedimentation Equilibrium. Sedimentation equilibrium experiments were carried out at rotor speeds of 12 000 and 17 000 rpm at 25 °C in a Beckman Optima XL-A centrifuge, with concentrations monitored by absorbance scans at 280 or 230 nm. Earlier studies of KGF by itself showed that it behaved as an ideal monomer at the sequence molecular mass. Samples of sKGFR by itself, and mixed at 1:1 or 2:1 molar ratios with KGF, were loaded at sKGFR concentrations of 1, 3, and 6 μM. The resulting data were analyzed through global fitting of 8−12 data sets, and the carbohydrate content of sKGFR was calculated from the data, using methods described previously (*41*, *42*).

RESULTS

Size Exclusion Chromatography (SEC) in the Presence of Heparin. While size exclusion chromatography is often used to investigate formation of protein complexes, its use in this case is complicated by the fact that KGF tends to bind to SEC resins and thus elutes at abnormal positions and with poor recovery, particularly at physiological ionic strengths. In our initial attempts, we failed to see formation of complexes between KGF and sKGFR, but it was unclear whether this truly indicated a lack of binding or was simply due to dissociation of complexes during chromatography due to loss of KGF and competition between its binding to the column matrix and its binding to the receptor.

This "stickiness" of KGF during SEC can be overcome by adding heparin to the elution buffer, presumably because the heparin binds to KGF (*43*). Therefore, the stoichiometry of complexes formed between KGF and sKGFR was investigated by mixing KGF and sKGFR in different molar ratios and analyzing resultant complexes by size exclusion chromatography in the presence of low-molecular mass (~3 kDa) heparin. The results are shown in Figure 2 for sKGFR and KGF when SEC was performed using a Superose 12 column with 30 μg/mL heparin 3000 added to the PBS elution buffer. When injected by itself, sKGFR eluted at a position before ovalbumin (44 kDa). Since its molecular mass as estimated from sedimentation equilibrium, which accounts for the polypeptide chain and the carbohydrate moiety, is 34 kDa (see below) this early elution position may indicate that it binds the heparin, but also could simply indicate an elongated shape in solution. The addition of KGF to sKGFR in the molar ratios noted in Figure 2 resulted in the appearance of a new peak eluting at a position slightly after γ-globulin (158 kDa) which most likely represents a sKGFR−KGF complex. When sKGFR and KGF are present at a 2:1 molar ratio during the incubation, only this new peak is detected and neither the free sKGFR peak nor the free KGF peak is present (Figure 2, chromatogram 5). Ligand added in excess of this ratio elutes as free KGF, with no further alteration of either the position or amplitude of the sKGFR−KGF complex peak (Figure 2, chromatograms 3 and 4). Receptor added in excess of this ratio elutes as free sKGFR, with no further alteration of either the position or amplitude of the peak corresponding to the sKGFR−KGF complex (Figure 2, chromatogram 6). Taken together, these results strongly suggest that the complex has a 2:1 molar

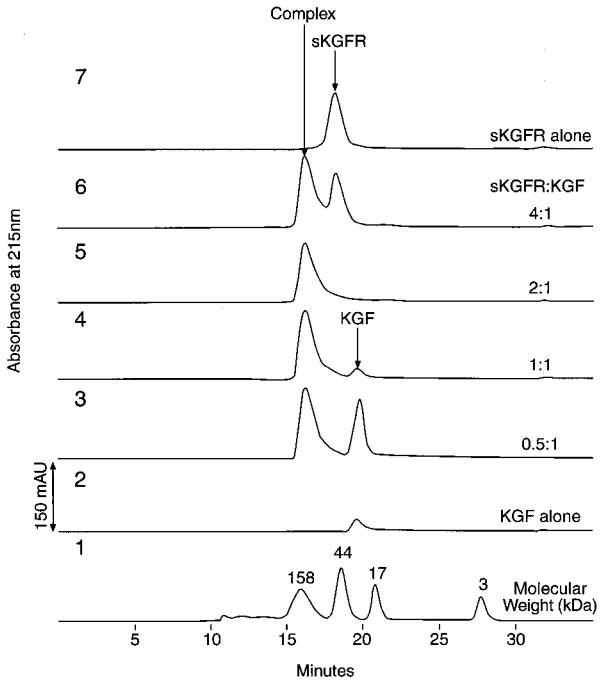

FIGURE 2: Superose 12 SEC experiments showing that sKGFR dimerizes upon KGF binding in the presence of heparin 3000 in the column buffer. sKGFR at 0.31 mg/mL (12 μM) was incubated with levels of KGF ranging from 0.1 to 0.4 mg/mL (6−24 μM), as well as 0.3 mg/mL heparin 3000 (0.1 mM). The molar ratios of sKGFR to KGF shown are 2:1, 1:1, and 0.5:1 (chromatograms 5−3, respectively). Chromatogram 2 represents data for KGF alone (6 μM), and chromatogram 7 represents data for sKGFR alone (12 μM). Incubations were carried out at room temperature for approximately 20−30 min, and aliquots (30 μL) of samples were then subjected to SEC, using a Superose 12 column as described in Experimental Procedures. Chromatogram 6 represents the data for sKGFR at 0.62 mg/mL (24 μM) incubated with KGF at 0.1 mg/mL (6 μM). Chromatogram 1 represents the data for molecular mass markers.

ratio of sKGFR to KGF. A similar conclusion can also be drawn from studies in which a silica-based column (TSK 3000SW-XL) was used when a large excess of heparin 3000 is added directly to the sample before injection but is not present in the elution buffer (data not shown).

N-Terminal Protein Sequencing. The molar ratio of 2:1 for the sKGFR−KGF complex was confirmed by N-terminal sequencing analysis. The fraction from Superose 12 SEC, representing the KGFR−KGF complex, was collected and sequenced. N-Terminal protein sequencing revealed that the ratio of initial sequence yield of sKGFR to KGF in the complex is 1.9, which closely matches the ratio of 1.8 obtained for the initial sequence yield of sKGFR to KGF by directly sequencing of a mixture of sKGFR and KGF made at a 2:1 molar ratio. The molar ratio of 2:1 for sKGFR to KGF in the complex could represent either 2:1 or 4:2 stoichiometry. Through comparison of the elution position of the complex to those of proteins with known molecular masses, the apparent molecular mass of the complex can be estimated as ~160 kDa, which is probably too low for a 4:2 stoichiometry given the fact that both free receptor and free KGF elute at positions implying anomalously high molecular masses. Therefore, to more accurately determine the stoichiometry for the complex, we used an on-line light scattering−SEC method.

Size Exclusion Chromatography with On-Line Light Scattering Detection and Refractive Index Detection. The molecular masses determined by on-line light scattering are independent of molecular shape and elution position. SEC in combination with three detectors (90° light scattering, UV absorbance, and refractive index) allowed us to calculate the polypeptide molecular mass of the sKGFR−KGF complex. Because the species concentrations are monitored by absorbance at 280 nm, a wavelength where the glycosylation on KGFR is not detected and where heparin is nearly undetectable, the signals from the three detectors may be combined in a way that algebraically cancels all contributions from glycosylation and heparin, such that the polypeptide molecular mass is obtained (the so-called "three-detector" method) (*44*). For these studies, we applied a sample containing 0.93 mg/mL (36 μM) sKGFR, 0.3 mg/mL KGF (18 μM), and 0.9 mg/mL (0.28 mM) heparin 3000 to a Superose 12 column, which produced a single major peak in all three detectors, corresponding to the sKGFR−KGF complex (Figure 3). This main peak has an identical shape in all three detectors, which implies that it represents only one species (only one molecular mass) throughout the whole peak. A small peak appearing at the void volume in the light scattering chromatogram, but not in the other two chromatograms, represents a trace amount of large protein aggregates. A broad peak around 22 min in the refractive index chromatogram is caused by heparin 3000 in the loading sample.

To calculate the polypeptide molecular mass of the complex, we need to know its extinction coefficient, which depends on the assumed stoichiometry (*41*, *44*). We therefore use a self-consistent approach in which the calculated molecular mass is deemed valid only if it is consistent with the stoichiometry assumed in calculating the extinction coefficient (Table 1). If we assume the complex contains one sKGFR and one KGF, the theoretical M_r equals 41 800 Da, which is inconsistent with the experimental value of 66 700 Da. If we assume the complex contains two sKGFR and two KGF, the theoretical M_r equals 83 600 Da, which is inconsistent with the corresponding experimental value of 66 700 Da. With an assumed stoichiometry of four sKGFR and two KGF for the complex, the theoretical M_r equals 134 600 Da, which is more than twice the experimental value of 65 000 Da. By contrast, for an assumed stoichiometry of two sKGFR and one KGF, the experimental M_r value of 65 000 Da agrees closely with the theoretical value of 67 300 Da. We therefore conclude that the complex indeed contains two sKGFRs and one KGF molecule.

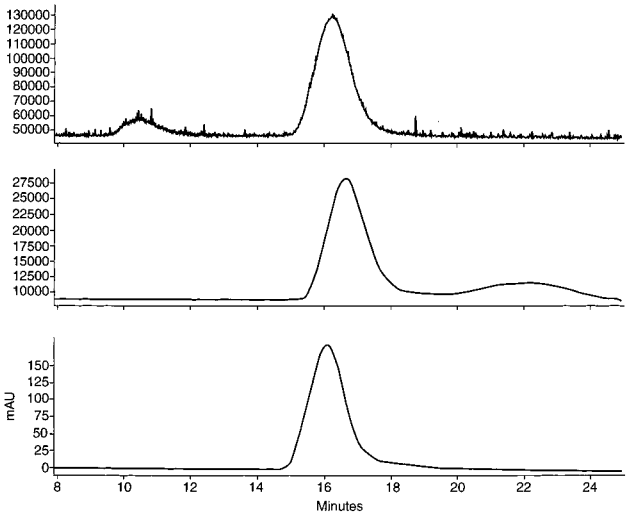

FIGURE 3: SEC using a Superose 12 column and three detectors showing that a 2:1 sKGFR−KGF complex was formed. The column was equilibrated with 2× PBS containing 30 μg/mL heparin 3000. The upper panel represents the light scattering trace, the middle panel the refractive index trace, and the lower panel the UV absorbance trace at 280 nm. There is a small delay between successive traces due to the interdetector volume.

Table 1: Summary of Light Scattering Results

assumed stoichiometry of the sKGFR−KGF complex	ϵ^a (mL mg^{-1} cm^{-1})	molecular mass from light scattering[a] ± 5% (kDa)	theoretical molecular mass[b] (kDa)	correct assumption?
1:1	1.49	67	41.8	no
1:2	1.44	69	58.1	no
2:1	1.53	65	67.3	yes
2:2	1.49	67	83.6	no
4:2	1.53	65	134.6	no

[a] Excluding carbohydrate. [b] Calculated from the sequence molecular masses.

Sedimentation Velocity. Further evidence for dimerization of sKGFR by a single KGF molecule was provided by sedimentation velocity studies. For these studies, a defined heparin decasaccharide fragment (3 kDa) was employed to reduce the molecular mass heterogeneity due to the heparin, as well as to eliminate longer heparin fragments that might be capable of oligomerizing KGF itself, as we have reported previously (*43*). The sedimentation velocity data were analyzed by time-derivative techniques to derive the sedimentation coefficient distribution (*40*). As shown in Figure 4, when sKGFR is mixed with KGF at a 2:1 molar ratio, in the presence of a 3-fold molar excess of heparin decamer over receptor, nearly all the protein in the mixture sediments as a complex with a sedimentation coefficient of 4.60 S,

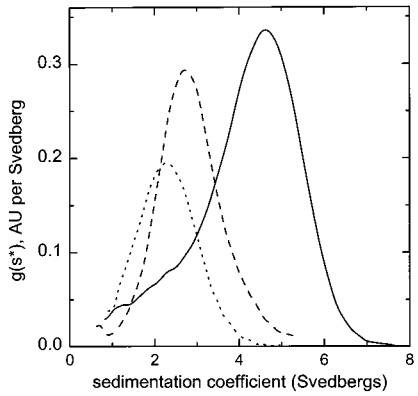

FIGURE 4: Sedimentation velocity experiments showing that sKGFR binds KGF in the presence of heparin decamer and a new species is formed with a sedimentation coefficient of 4.60 S. The sedimentation coefficient distribution function, $g(s^*)$, measures the amount of material (as monitored by absorbance at 230 nm) sedimenting at each sedimentation coefficient. The dotted line represents data for a sample containing 2 μM KGF and 6 μM heparin decamer; the dashed line represents data for a sample containing 2 μM sKGFR and 6 μM heparin decamer, and the solid line represents data for a sample containing 2 μM sKGFR, 1 μM KGF, and 6 μM heparin decamer.

whereas when sKGFR and KGF are run separately in the presence of the same amount of heparin, they sediment at 2.74 and 2.28 S, respectively. The high sedimentation coefficient of the complex, and the fact that there is little free sKGFR or KGF present in the mixture, both imply that this complex contains two molecules of KGFR and one KGF. Furthermore, a similar experiment where additional KGF was added to make an equimolar mixture of sKGFR and KGF again gave a peak at 4.6 S but also a peak at 2.28 S corresponding to excess KGF (not shown), again confirming the 2:1 stoichiometry. The sedimentation coefficients of sKGFR and KGF alone are both slightly higher in the presence of the heparin decamer than when it is absent (data not shown), indicating that both proteins can bind this heparin fragment, but that neither is dimerized by it.

Because sedimentation coefficients depend on the shape and degree of hydration in addition to molecular mass, and because the glycosylation of the receptor has a disproportionately large effect on its hydrodynamic properties, it is not possible to derive an accurate molecular mass for the complex from its sedimentation coefficient. However, if we use a simple spherical approximation, then the ratio of molecular masses implied by the sedimentation coefficient for the complex relative to receptor plus heparin is ~2.2, in reasonable agreement with the 2.4-fold ratio calculated for two receptors, KGF, and one decamer (~79 kDa) versus one receptor and one decamer (~33 kDa). Thus, the sedimentation coefficient of the complex is consistent with a 2:1 stoichiometry, but is far too low to be consistent with a 4:2 stoichiometry.

These sedimentation velocity studies do not directly determine the affinities for these interactions. However, the almost complete shift of the sedimentation coefficient distribution for samples made at 2:1 molar ratios to a peak representing the 2:1 complex, and the fact that the position of this peak does not shift when excess KGF is added, together imply that the dissociation constants are far smaller than the micromolar concentrations. A comparison to simulations of sedimentation velocity experiments carried out by

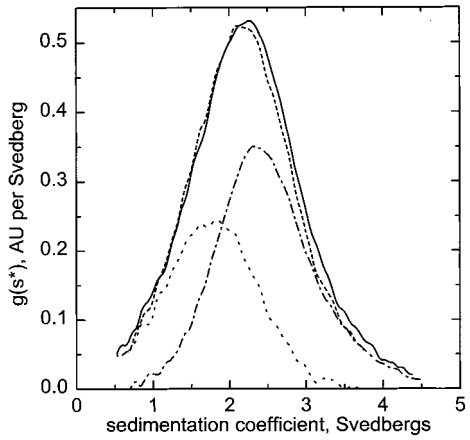

FIGURE 5: Sedimentation velocity data showing that sKGFR does not bind KGF in the absence of heparin. The sedimentation coefficient distribution of a mixture of 2 μM sKGFR and 2 μM KGF (solid line) is identical, within error, to the arithmetic sum (dashed line) of the data for each protein run separately. The dotted line represents data for 2 μM KGF alone; the dashed−dotted line represents data for 2 μM sKGFR alone.

Stafford (*45*) allows an order-of-magnitude estimate of the *maximum* value for the K_d's of the receptor−KGF interactions of 10^{-8}−10^{-9} M. Similarly, the shift of the sedimentation coefficients of KGF and sKGFR when run in the presence of the heparin decamer implies that each has a K_d for the decamer of less than ~1 μM.

We are unable to determine from these data the number of heparin decamers in the complex. Similar experiments with a 2:1:1.1 sKGFR:KGF:heparin ratio (not shown) gave about two-thirds of the protein sedimenting as a broad peak around 4.3 S as well as some uncomplexed proteins, suggesting that only a single decamer is required to promote receptor dimerization, and that the affinity of the heparin interactions is insufficient to maintain full complex formation at the 1.1 μM heparin concentration in that experiment.

The results of similar studies in the absence of heparin are strikingly different. Figure 5 shows the analysis for a mixture containing 2 μM sKGFR and 2 μM KGF. The sedimentation coefficient distribution of this mixture is identical, within error, to the arithmetic sum of data for each protein run separately, strongly implying that there is no tight binding between these proteins in the absence of heparin. While these data cannot rule out the possibility that binding would occur at higher protein concentrations, they certainly imply that the dissociation constant must be greater than 10 μM, i.e., more than 5 orders of magnitude weaker than the affinities reported for the receptor on the surface of cells (where heparan sulfate or similar proteoglycans are presumably present).

Heparin Length Requirement for Complex Formation. To assess the minimum length of heparin oligosaccharide that promotes effective receptor dimerization, sedimentation velocity studies were also carried out using heparin hexamers and octamers. Control studies show that sKGFR and KGF when run separately will each bind these shorter heparin fragments at a concentration of 6 μM. Data for ternary mixtures using either the hexamer, octamer, decamer, or conventional heparin 3000 are summarized in Figure 6. The octamer, decamer, and heparin 3000 all give predominantly the 2:1 complex sedimenting at 4.4−4.6 S, whereas the

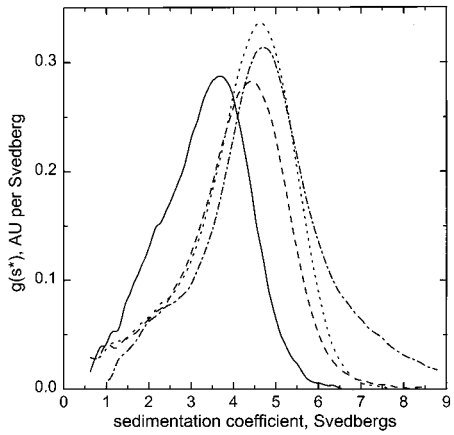

FIGURE 6: Heparin length requirement for formation of 2:1 receptor−ligand complexes. The sedimentation coefficient distributions were determined as described in the legend of Figure 4, except the length of the heparin fragment was varied. All samples contained 2 μM sKGFR, 1 μM KGF, and 6 μM heparin fragment: (solid line) heparin hexamer, (dashed line) heparin octamer, (dotted line) heparin decamer, and (dash−dotted line) heparin 3000. Even though the defined decamer and the heparin 3000 have the same nominal average molecular mass, the heparin 3000 gives minor species larger than 6 S, which probably arise from minor higher-molecular mass heterogeneous heparin fragments which are long enough to allow binding of additional proteins on the heparin molecule.

hexamer produces a peak at 3.6 S. This 3.6 S peak might represent a 1:1 sKGFR−KGF complex, but it could also arise from weaker binding of the hexamer if that gives a rapid association and dissociation of 2:1 complexes, thereby producing an apparent single peak at an intermediate sedimentation coefficient.

Sedimentation Equilibrium in the Absence of Heparin. To further confirm that heparin is required for strong binding of KGF to sKGFR, we also studied mixtures of sKGFR and KGF by sedimentation equilibrium, a true equilibrium method that is capable of detecting much weaker interactions than can be detected by SEC. Control studies of each protein by themselves showed them to be monomeric. Using the sequence molecular mass of 25 300 Da for sKGFR, the sedimentation data imply a total molecular mass (including glycosylation) of 32 900 ± 900 Da (23 ± 3% carbohydrate by weight).

The data for 2:1 and 1:1 mixtures of sKGFR with KGF in PBS (no heparin) at loading concentrations of up to 6 μM show very little binding, and no evidence for any 2:1 complexes, consistent with the sedimentation velocity studies. Figure 7 depicts some results for a 1:1 mixture. The slope of the data is only slightly above that expected if there is no binding, and well below that for a tight 1:1 complex. This clearly shows that the binding is at best very weak, even though the protein concentration is >5 orders of magnitude higher than the K_d's reported for binding to receptors on cells. The detailed analysis of these sedimentation equilibrium experiments is complicated by the instability of the samples over the long time periods necessary for these studies, and we clearly see evidence for formation of some large aggregates over time. Given the 2:1 stoichiometry seen when heparin is present, we tried globally fitting 12 data sets to models where KGF has two binding sites for sKGFR, using the methods we have previously applied to quantitate the interactions of other bivalent growth factors such as neurotrophin-3, stem cell factor, and erythropoietin with their

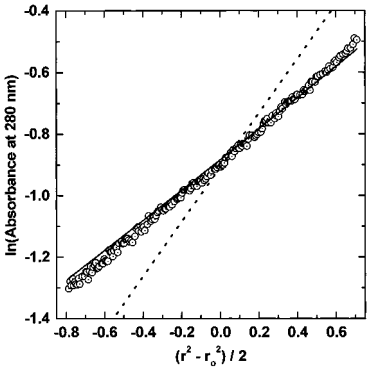

FIGURE 7: Sedimentation equilibrium data for a mixture of 6 μM sKGFR and 6 μM KGF in PBS. When the data are plotted in this fashion, a single species will give a straight line whose slope is proportional to molecular mass. The solid line describes the predicted data if there is no interaction between these proteins, based on control studies of each protein by itself. The dotted line shows the slope predicted for a 1:1 complex. Data were recorded after 24 h at 12 000 rpm.

soluble receptors (*46*−*48*). These models with two binding sites will not converge, because during fitting the affinity of one of the sites approaches zero. Given this evidence that in the absence of heparin the second binding site of KGF is absent or extremely weak, we therefore tried a model with a single binding site, which provides a fairly good fit when that site has a K_d of 70 ± 20 μM. Because the samples contain some aggregates, which will bias the fit toward stronger association, we regard this 70 μM figure as a lower limit for the K_d, and the true binding affinity may well be even weaker. In summary, these sedimentation equilibrium studies show no evidence for receptor dimerization in the absence of heparin. Further, although some 1:1 complexes can form at high protein concentrations, the binding affinity is so weak that no binding to receptor would occur at physiologically relevant concentrations of KGF.

KGF- and Heparin-Dependent DNA Synthesis and Cell Growth Assays. Biophysical and chemical data described earlier indicate heparin is essential for KGF-induced KGFR dimerization. To examine whether this heparin requirement also applies to KGFR on the cell surface, we employed an IL-3-dependent murine myeloid cell line 32D, which does not express endogenous KGFR or low-affinity FGF binding sites (*24*), and transfected it with a chimeric receptor consisting of the three Ig-loop extracellular ligand binding domain of KGFR and the transmembrane and intracellular domains of the murine EPOR. Heparin was found to dramatically improve KGF stimulation of 32D cells containing the KGFR−EPOR chimera (Figure 8). Only 10 ng/mL KGF was required to reach 50% IL-3 maximal stimulation in the presence of heparin, whereas in the absence of heparin, 1000 ng/mL KGF was required. As a negative control, the 32D cells transfected with vector alone showed no stimulation by KGF in the presence or absence of heparin (data not shown). Heparin was found to enhance the activity of KGF by more than 100-fold. The concentration of KGF required for signal transduction in the absence of heparin exceeded what would be expected to be found under normal physiological conditions and may be explained by the participation of suboptimal glycosaminoglycans on the 32D cell surface.

We also examined the heparin dose-dependent proliferation response of 32D KECA cells using a fixed concentration of

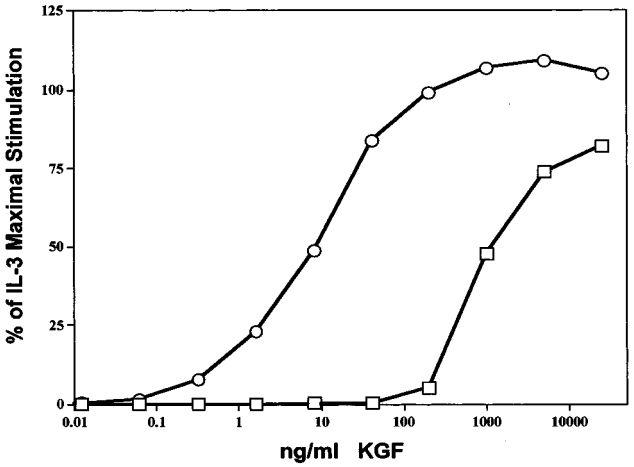

FIGURE 8: KGF dose-dependent stimulation of DNA synthesis in 32D KECA cells. KGF-stimulated [^{3}H]thymidine uptake is expressed as a percentage of maximal stimulation (IL-3) minus background (no addition). 32D KECA cells were washed free of IL-3 and then incubated with the indicated doses of KGF for 48 h with heparin (○) and without heparin (□). The concentration of heparin used here was 500 ng/mL. The concentration of IL-3, used for the maximal stimulation as a reference control, was 10 ng/mL. The data represent the average of triplicate values, and the standard deviation in the assay was ±5%.

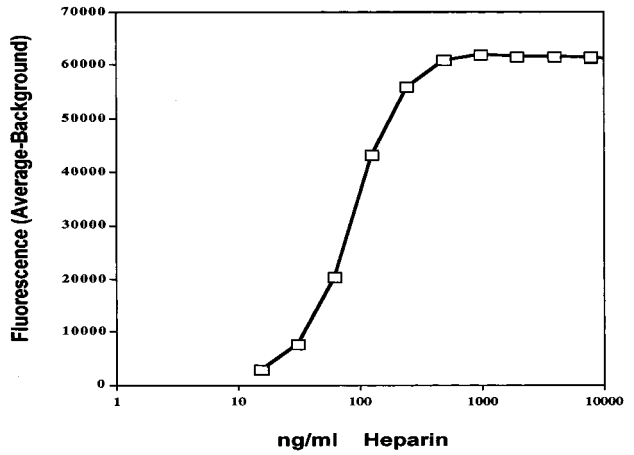

FIGURE 9: Heparin dose-dependent proliferation in 32D KECA cells. 32D KECA cells were washed free of IL-3 and then incubated with 12.5 ng/mL KGF and a varied concentration of heparin for 48 h. Alamar blue was added 48 h after the initiation of culture. The plates were read after an additional 24 h. The background was measured at no addition of KGF and heparin. The data represent the average of triplicate values, and the standard deviation in the assay was ±5%.

KGF at 12.5 ng/mL (Figure 9). A half-maximal response required approximately 80 ng/mL heparin. While these proliferation experiments were performed using a heparin with a molecular mass of 16 000 Da, comparable results were obtained with lower-molecular mass (3000 Da) heparin (data not shown). These results indicated heparin is essential for KGF stimulation of DNA synthesis and cellular proliferation of 32D KECA cells.

DISCUSSION

With a variety of biophysical and chemical methods, including size exclusion chromatography (SEC), light scattering, sedimentation velocity, and N-terminal protein sequencing, we have clearly shown that KGF forms a complex with the sKGFR in the presence of heparin, and that this complex has a 2:1 stoichiometry of KGFR to KGF. KGF was found to interact with the column matrix in the absence of heparin, and thus, it was not entirely clear from these SEC studies whether heparin is required for formation of the complex or whether it merely prevents dissociation of the complex during chromatography by reducing the extent of interaction of KGF with the column matrix. This ambiguity has been completely resolved, however, by the analytical ultracentrifugation studies. These showed that in the absence of heparin, no KGFR−KGF complex was detected at the concentrations of 1−2 μM that were used in the sedimentation velocity experiments. Sedimentation equilibrium experiments at higher protein concentrations in the absence of heparin showed a very weak interaction (K_d > 70 μM) in forming a 1:1 complex, but no evidence of a 2:1 receptor−ligand complex.

In contrast, sedimentation velocity studies demonstrated that at 1−2 μM protein concentrations but in the presence of heparin octamer or decamer, sKGFR binds KGF, and a new species is formed with a sedimentation coefficient of 4.4−4.6 S. This value, as well as the disappearance of the peaks for free sKGFR and KGF, strongly implies formation of a 2:1 receptor−KGF complex and supports the results of experiments with size exclusion chromatography. The sedimentation studies clearly establish that heparin is absolutely required for receptor dimerization and for formation of a tight complex. These studies also provided preliminary evidence that the heparin must be at least eight saccharides long to promote formation of a stable 2:1 complex. A more complete definition of the heparin structural requirements must await studies in which more structurally homogeneous heparin fragments will be used as well as characterization of the binding affinities of those fragments.

These studies also firmly establish that the intrinsic affinity of KGF for its receptor (in the absence of heparin) is actually significantly weaker than the affinities that both proteins have for heparin. This, and the fact that cells responsive to KGF have very high levels of HSPG sites, would suggest it is quite unlikely that the first step in receptor activation in vivo is KGF binding to receptor. Rather, an interaction of either the receptor or KGF with heparin or heparan sulfate probably occurs prior to the binding of KGF to receptor. The fact that in solution 2:1 sKGFR−KGF complexes are favored over 1:1 complexes also suggests that once KGF binds to a single receptor or receptor−HSPG complex on a cell, the binding of a second receptor will be very strongly favored, particularly given the favorable entropic effects of confining the receptors within the cell membrane.

Although in several cell-based studies researchers attempted to examine the heparin dependency of the KGF−KGFR interaction by either removing HSPG sites by treatment with heparinases or heparitinase or reducing the extent of sulfation by treatment with chlorate (*26*, *28*), the complexity of these systems and uncertainties about whether all HS is removed by such treatments did not allow a definitive assessment of the issue. The present results both confirm and extend the recent studies of Jang et al. (*30*), who demonstrated that heparin is required for KGF binding and activity on keratinocytes whose HS was blocked by protamine sulfate. We have also shown using a KGFR−EPOR chimera expressed in 32D myeloid cells that heparin is required to achieve maximal stimulation by KGF (Figures

8 and 9). In the absence of heparin, very high concentrations of KGF were necessary to stimulate the 32D cells expressing the KGFR−EPOR chimera, and these concentrations of KGF are not physiologically relevant. Since receptor dimerization has been shown to be required for EPOR signal transduction, these studies suggest that heparin is facilitating KGF binding to the KGFR and dimerization of the receptor on the surface of the 32D cells. 32D cells are known to lack low-affinity FGF binding sites (HSPGs), and previous studies in which bFGF and 32D cells transfected with FGFR2 were used have also determined that heparin is required for bFGF binding and stimulation of growth of these cells (*24*). While the KGFR chimera on the surface of these 32D cells in our studies includes the extracellular domain of a three-Ig-like loop isoform of human KGFR, the studies of KGFR in solution were performed with a soluble KGFR comprising two Ig-like loops (II and III) of the extracellular domain. There are naturally occurring isoforms of human KGFR with either two (II and III) or three Ig-like domains (*49*), and the KGFR expressed on mouse keratinocytes has only two Ig-like domains (*15*). Our results demonstrate a heparin requirement for both two- and three-Ig-like loop isoforms of KGFR.

The epithelial cells shown to respond mitogenically to KGF also possess high numbers of HSPG sites, and it is now clear that these probably play a key role in modulating their response to KGF as well as their differential response to other FGFs. Heparan sulfate proteoglycans are complex macromolecules with differing lengths and chemical compositions, and most cells present a variety of HS species. The FGF ligands are thought to exhibit different affinities for differing types of glycosaminoglycan (GAG). For example, it has been shown that the chemical composition of the disaccharide repeat required for bFGF to interact with receptor is different than that required by aFGF (*50*, *51*). In our studies, we have found that heparin (which is more highly sulfated than heparan sulfate) is more efficient than heparan sulfate in promoting the KGFR−KGF interaction (data not shown). Other than the ability of HSPG sites to promote the FGFR−FGF interaction, HSPG sites on the cell surface are thought to serve as "ligand reservoirs" in which FGFs bind and are stabilized against misfolding (*52*, *53*) and/or proteolysis (*54*, *55*). Therefore, the level of biologically active FGF available to cells is likely to be controlled by both the half-life or stability of the FGF and the strength of the FGF interaction with its FGFR. Both of these parameters are likely to be affected by glycosaminoglycans.

In an intensive study by Pantoliano et al. (*33*) in which highly purified and defined components were used, they similarly have found that heparin is required to form a 2:1 complex of sFGFR1 with bFGF. The distinct difference between the sKGFR−KGF system and the sFGFR1−bFGF system is that KGFR and KGF do not form a strong 1:1 receptor−ligand complex in the absence of heparin. For sFGFR1 and bFGF in the absence of heparin, a 1:1 complex forms with a reported K_d of 20 nM (*33*), whereas for sKGFR and KGF, this K_d is >70 μM. Our sedimentation velocity studies also established that sKGFR and KGF can each interact with heparin in the absence of the other protein. Although Pantoliano et al. (*33*) also observed that both sFGFR1 and bFGF interact with heparin, an important difference between these systems is that sKGFR appears to interact with heparin significantly more strongly than does FGFR1, where the affinity is only ~100 μM (*33*). A region N-terminal to Ig-like loop II of FGFRs has been shown to constitute a heparin binding site (*11*, *56*), and the interaction of heparin with this site has been suggested to be obligatory for the formation of a bioactive FGF−FGFR complex (*56*). This site is highly conserved, and most known functional FGFR2 splice variants contain this site (*11*), including ours (see Figure 1).

The X-ray crystal structure of bFGF with a heparin hexamer has been determined (*57*). The crystal structure showed that the heparin binding pocket of bFGF can hold a heparin hexamer fragment, and the overall bFGF crystal structure was found to be similar to the structure determined previously in the absence of heparin, suggesting that no major conformational changes of bFGF occur upon heparin binding (*57*). This result and the observation by Pantoliano et al. (*33*) that bFGF forms a 1:1 complex with sFGFR1 in the absence of heparin would seem to rule out the previously proposed induced-fit model (*22*) that suggests that bFGF must first interact with heparin and then undergoes a conformational change that allows it to bind receptor. It should be remembered, however, that our studies and those of Pantoliano et al. (*33*) both show that the receptor also interacts with heparin. Kan et al. (*56*) have suggested that the FGFR1 interaction with heparin is required for formation of a complex of bFGF with the FGFR. Therefore, a possibility that the receptor may undergo a conformational change upon interacting with heparin remains. Although the heparin interaction with receptor generally exhibits a lower affinity than that with ligand, cells that express FGFRs typically also express HSPGs in the range of 10^5-10^6 copies per cell (*19*), and therefore, the interaction of these sites with receptor is most likely biologically relevant (*33*). In the case of the KGFR−KGF−heparin complex, the KGFR interaction with heparin is much stronger than the FGFR1 interaction with heparin, the KGF interaction with KGFR in the absence of heparin is much weaker than the bFGF−FGFR1 interaction, and the interaction of KGF with heparin is weaker than what has been reported for bFGF. Thus, it is likely that in vivo the order of assembly of active signaling complexes differs between KGFR and FGFR1.

The driving force for KGF receptor dimerization can be explained in terms of allosteric multivalent binding reactions that allow the cooperative energetic coupling of heparin binding reactions on KGFR and KGF with receptor−ligand binding events. When the minimum free energy state is reached, the stable 2:1 sKGFR−KGF complex with heparin is formed. Our results conflict with models proposing that receptor dimerization is due to ligand dimerization by heparin or HS molecules, with this ligand dimer then promoting formation of a 2:2 FGFR−FGF complex (*9*, *23*, *32*, *58*). We find the true stoichiometry of sKGFR−KGF in our study is 2:1, as is the stoichiometry reported earlier for sFGFR1−bFGF (*33*). Further, it is quite significant that the heparin octamer and decamer fragments are capable of promoting strong, stable 2:1 complexes, but do not dimerize either KGF or the receptor at the concentrations used in these sedimentation velocity studies.

In recent years, the ligand dimerization model has received further support from NMR and X-ray structures of FGF dimers and tetramers induced by heparin fragments *(58*−*60*). It is therefore worth discussing (1) whether it is possible

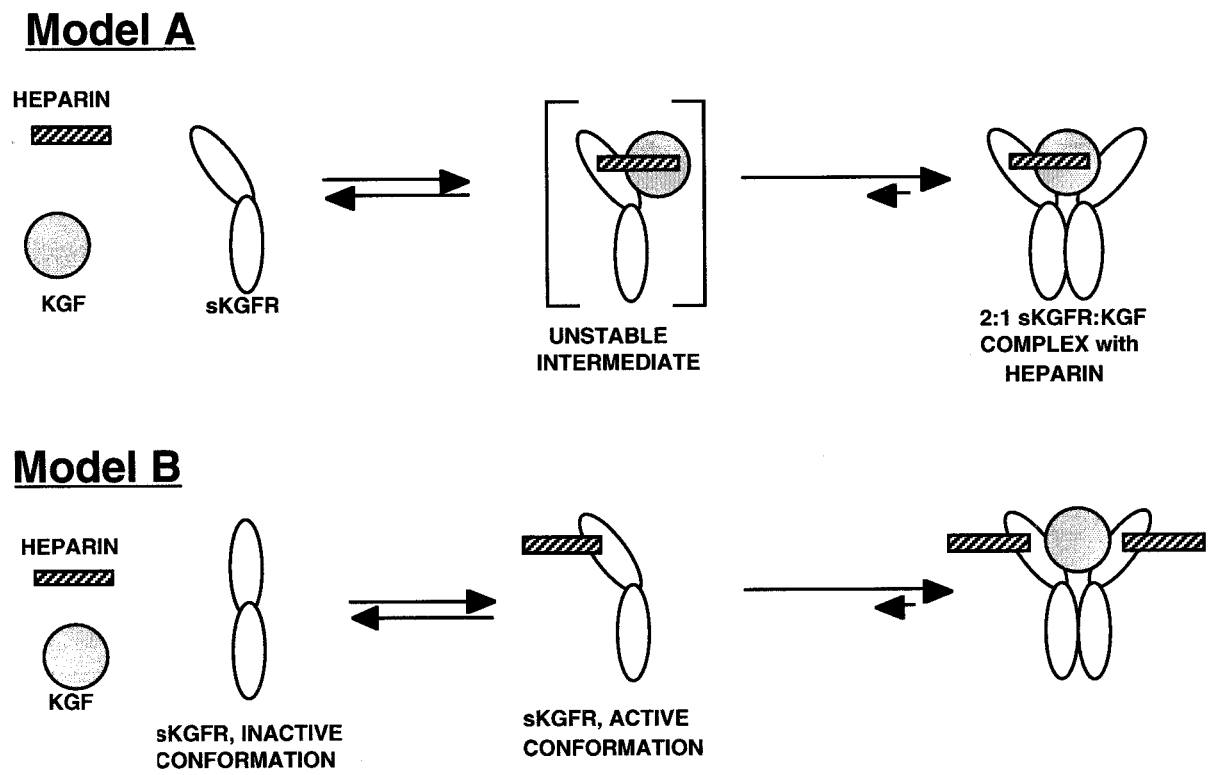

FIGURE 10: Schematic diagram of a model of KGFR receptor dimerization by KGF and heparin (or HS). Model A depicts a sequential binding mechanism, somewhat analogous to that proposed for human growth hormone (*66*). In the first step, heparin facilitates the interaction of KGFR with KGF to form an unstable intermediate 1:1:1 complex. This complex in turn provides a surface (possibly involving receptor−receptor contacts) to which a second receptor can bind to form the more stable 2:1 KGFR−KGF complex. Although heparin is shown cross-bridging only one receptor and ligand molecule, it is possible that a single heparin molecule of sufficient length could simultaneously interact with two KGFR molecules and KGF, thus spanning all three proteins in the 2:1:1 complex. In model B, heparin is required to stabilize the "active" conformation of the receptor. That active conformation is capable of binding to two different sites on KGF. The location of the heparin binding site and the nature of the conformational change shown here are strictly schematic; the heparin binding site is actually thought to be near the N-terminus of this sKGFR construct (see Figure 1) (*11*, *56*). Although it is not shown here, heparin might also be bound to the KGF molecule in the complex.

to reconcile the present results with those studies and (2) whether the receptor dimerization mechanisms may actually differ among members of the FGF family. The structural studies show the existence of two fundamentally different types of dimers: a cis dimer with two FGFs side by side along the length of the heparin and a trans dimer with the heparin sandwiched between two FGF molecules located on opposite sides of the heparin helix (*60*). Symmetric tetramers containing both cis and trans dimers have also been reported (*58*). Given that these heparin-induced dimers clearly do exist, one possible way to reconcile the 2:1 molar ratios seen both here and by Pantoliano et al. (*33*) might be if the complexes were actually 4:2 complexes rather than 2:1. However, for sKGFR and KGF both the light scattering and sedimentation velocity data strongly rule out a putative 4:2 stoichiometry.

In our view, the key question about the heparin-induced FGF oligomers is whether they actually exist at physiologically relevant concentrations. For example, the bFGF tetramers induced by a heparin decamer were observed in NMR experiments at protein concentrations of 1 mM (16 mg/mL). However, earlier sedimentation equilibrium studies showed that the bFGF dimers and tetramers induced by a heparin octamer dissociate to monomers at concentrations below ~100 μM (*61*), so the physiological relevance of those bFGF oligomers is certainly open to question.

With regard to possible fundamental differences between FGF family members, we believe the evidence suggests that aFGF may indeed differ significantly from bFGF and KGF. Studies with a soluble form of FGFR2 by Spivak-Krolzman et al. (*62*) showed that in the absence of heparin it binds to aFGF but forms only 1:1 complexes. In the presence of heparin, these 1:1 complexes dimerized to form 2:2 complexes (*62*). Therefore, the stoichiometry does appear to be different than that for FGFR1−bFGF (*33*) and KGFR−KGF; however, we note that in those studies the receptor was never present in excess over aFGF, and thus, a 2:1 stoichiometry might exist under different conditions. One significant difference is that FGFR2 apparently does not itself interact strongly with heparin (*62*), unlike KGFR (as shown here) and FGFR1 (*33*). A second important difference is that at protein concentrations of $\leq$1 mg/mL approximately twice as many aFGF molecules will bind to a heparin molecule of a given size (5−16 kDa) as will bFGF or KGF molecules (*43*, *62*−*64*). This observation is consistent with aFGF having a much stronger tendency to form trans dimers with heparin than does bFGF or KGF. If such trans dimers are incapable of binding a second receptor molecule to the same ligand (perhaps due to steric considerations), then a trans dimer would form 2:2 rather than 4:2 complexes. If this is true, the relative tendency of a given FGF type to form trans or cis dimers with heparin would explain the different stoichiometries of receptor complexes (and perhaps provide another means of regulating activation and specificity toward different receptor subtypes).

These new results for KGF and KGFR are compatible with a number of alternative models for receptor activation. Our results would be consistent with a model where heparin is required to cross-bridge KGFR and KGF to form an initial unstable intermediate 1:1 complex (Figure 10, model A), thus creating an interface to which a second KGFR can bind to form the more stable 2:1 complex. Such a cross-bridging model would also fairly easily explain why we find that heparin octamer or decamer is sufficiently long to give a stable 2:1 complex, but a heparin hexamer is not. Although this 2:1 complex is shown for simplicity to be symmetrical, there is no known symmetry within KGF and its two interactions with the receptor would presumably involve different residues and have different binding affinities. In accordance with this, Springer et al. (*65*) have provided data that show that monomeric bFGF mediates receptor dimerization by utilizing two separate FGFR binding sites on opposite faces of the same bFGF molecule. The primary, higher-affinity binding surface of bFGF was found to have an ~250-fold higher affinity for FGFR than the secondary binding surface. Our data could, in addition, also be explained by models invoking heparin-driven conformational changes in the receptor (*11*), such as that shown as model B in Figure 10. Also, the existing data could be explained by a combination of models A and B in which heparin induces a conformational change in KGFR to form an active conformation of receptor (model B) and heparin also allows formation of a cross-bridge between ligand and receptor (model A). We are hopeful that future studies will provide data that will distinguish among these possibilities.

The current studies, together with the studies of the FGFR1−bFGF system by Pantoliano et al. (*33*), suggest that the formation of a 2:1 receptor−ligand complex and its mediation by heparin or HSPGs occur for at least two members of the FGF family and may be a requirement for signal transduction in vivo. The 2:1 stoichiometry strongly suggests that each FGF ligand contains two binding sites for receptor, as proposed for bFGF by Springer et al. (*65*). Finally, the almost complete absence of KGF binding to receptor in the absence of heparin and the relative inability of KGF to stimulate 32D cells containing the KGFR−EPOR chimera in the absence of heparin both demonstrate even more strongly the central role of HSPGs in governing and regulating the activity of the FGF family of ligands.

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