Heparan Sulfate Proteoglycan Modulates Keratinocyte Growth Factor Signaling through Interaction with both Ligand and Receptor

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ABSTRACT: Keratinocyte growth factor (KGF) is an unusual fibroblast growth factor (FGF) family member in that its activity is largely restricted to epithelial cells, and added heparin/heparan sulfate inhibits its activity in most cell types. The effects of heparan sulfate proteoglycan (HSPG) on binding and signaling by acidic FGF (aFGF) and KGF via the KGFR were studied using surface-bound and soluble receptor isoforms expressed in wild type and mutant Chinese hamster ovary (CHO) cells lacking HSPG. Low concentrations of added heparin (1 μg/mL) enhanced the affinity of ligand binding to surface-bound KGFR in CHO mutants, as well as ligand-stimulated MAP kinase activation and *c-fos* induction, but had little effect on binding or signaling in wild type CHO cells. Higher heparin concentrations inhibited KGF, but not aFGF, binding and signaling. In addition to the known interaction between HSPG and KGF, we found that the KGFR also bound heparin. The biphasic effect of heparin on KGF, but not aFGF, binding and signaling suggests that occupancy of the HSPG binding site on the KGFR may specifically inhibit KGF signaling. In contrast to events on the cell surface, added heparin was not required for high-affinity soluble KGF−KGFR interaction. These results suggest that high-affinity ligand binding is an intrinsic property of the receptor, and that the difference between the HSPG-dependent ligand binding to receptor on cell surfaces and the HSPG-independent binding to soluble receptor may be due to other molecule(s) present on cell surfaces.

Keratinocyte growth factor (KGF)[1] is a member of the fibroblast growth factor (FGF) family, also known collectively as heparin-binding growth factors. KGF regulates aspects of embryonic development and adult homeostasis by stimulating cell migration, proliferation, differentiation, and cytoprotection (*1*−*3*). Unlike most FGFs, KGF is produced by cells of mesenchymal origin but acts primarily on cells of epithelial origin through a specific receptor tyrosine kinase encoded by an alternative transcript of FGF receptor-2 (FGFR-2; *1*, *4*−*6*). In addition to its role as a mediator of normal mesenchymal−epithelial interaction, KGF has been implicated in certain pathological conditions, such as inflammatory bowel disease (*7*, *8*), benign prostatic hypertrophy, and prostate cancer (*9*−*11*). The widespread involvement of KGF in development, homeostasis, and disease provides a strong impetus for uncovering the structural basis of KGF signaling, and the role of cell-surface heparan sulfate proteoglycan (HSPG) in this process.

Immobilized heparin greatly facilitated the initial purification of FGFs, and soluble heparin/heparan sulfate is a potent modulator of FGF activity in model cell systems (*12*, *13*). HSPG present on most cell surfaces and in extracellular matrixes can protect FGFs from thermal denaturation and proteolytic attack, and may act as a protective reservoir where FGF release occurs through extracellular matrix turnover (*14*, *15*). Studies on cells expressing high-affinity FGFRs in the absence of HSPG demonstrate loss or attenuation of FGF responsiveness that can be restored with added soluble heparin, suggesting that HSPG may enhance (*16*) or be required for (*17*−*19*) FGF binding and signaling. The heparin and heparan sulfate binding domains of acidic FGF (aFGF) and basic FGF (bFGF) have been localized by biochemical and molecular genetic techniques (*20*−*23*), and by X-ray crystallography (*24*−*30*).

The recent cocrystallization of a dimeric biologically active aFGF−heparan sulfate complex provides a structural basis for receptor kinase dimerization and transactivation (*30*).

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[1] Abbreviations: KGF, keratinocyte growth factor; FGF, fibroblast growth factor; KGFR, KGF receptor; FGFR, FGF receptor; HSPG, heparan sulfate proteoglycan; CHO, Chinese hamster ovary; HPLC, high-performance liquid chromatography; PBS, phosphate-buffered saline; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PVDF, polyvinylidine difluoride; BSA, bovine serum albumin; DMEM, Dulbecco's Modified Eagle's Medium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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However, the demonstrated interaction of heparin and heparan sulfate with FGFR-1 adds another level of complexity to the role of HSPG in FGF signaling, particularly since deletion of the putative HSPG binding region within the receptor extracellular domain also resulted in the loss of FGF binding and signaling (*31*). Although it is not yet known whether this is a universal feature of FGFRs, it suggests that HSPG may bind both ligand and receptor, and that each of these events somehow regulates receptor dimerization, activation, and signaling.

We have shown previously that KGF binds with low affinity to HSPG in mouse keratinocytes (*32*), and that added soluble heparin can inhibit KGF-stimulated DNA synthesis in that setting (*33*). To further characterize the role of HSPG in KGF binding and signaling, we used both surface-bound and soluble receptor isoforms expressed ectopically in wild type and mutant Chinese hamster ovary (CHO) cells lacking HSPG. Like that of other FGFs previously examined, KGF binding and signaling in cells lacking HSPG is dramatically enhanced by low concentrations of added soluble heparin. However, in all cell types tested, higher heparin concentrations inhibited KGF, but not aFGF, binding and signaling through the KGFR. In contrast, added heparin was not required to observe high-affinity ligand binding to soluble KGFR expressed by either wild type or mutant CHO cells. The apparent need for HSPG to facilitate ligand binding to cell-surface-bound receptor suggests the presence of a negative modulator of ligand binding in that environment, and that HSPG may function to displace this modulator and in turn facilitate ligand binding and signaling.

EXPERIMENTAL PROCEDURES

Materials. Recombinant human KGF and [^{125}I]KGF were prepared as described previously (*32*). Bovine aFGF was obtained from Upstate Biotechnology (Lake Placid, NY) or prepared and radiolabeled as reported previously (*34*). Protein A-Sepharose CL-4B, GammaBind G-Sepharose, Sepharose CL-6B, and heparin−Sepharose were purchased from Pharmacia LKB Biotechnology, Inc. (Piscataway, NJ). [^{3}H]Heparin (specific activity of 440 μCi/mg) was purchased from NEN (Boston, MA). Disuccinimidyl suberate (DSS) and Nonidet P-40 (NP40) were obtained from Pierce Chemical Co. (Rockford, IL). Heparin purified from porcine intestine, which contains a mixture of heparin and heparan sulfate, was purchased from Sigma (St. Louis, MO).

Cell Culture, cDNA Constructs, Transfection, and Metabolic Labeling. Balb/MK mouse keratinocytes were maintained as described previously (*1*). Wild type CHO cells were obtained from the American Type Culture Collection (Rockville, MD). The mutant CHO cell line pgsA-745 (CHO-745) was kindly provided by J. D. Esko. CHO cells were maintained in Ham's F12 medium containing 10% fetal bovine serum.

Rat KGFR cDNA (*35*) expression constructs were generated using the pCEV27 expression vector (*5*). Stable, G418-resistant CHO KGFR transfectants were generated using Lipofectin (Life Technologies) according to the manufacturer's instructions.

A chimeric molecule containing the extracellular domain of the mouse KGFR fused in-frame to the constant region of the mouse IgG heavy chain (HFc) was constructed, expressed, and purified by protein A-Sepharose chromatography as described previously (*36*). The identity of the KGFR−HFc protein on SDS−PAGE was confirmed by immunoblotting with a KGFR-specific antisera (data not shown). The KGFR−HFc protein was metabolically labeled by incubating transfectants in medium containing [^{35}S]-methionine and [^{35}S]cysteine for 4 h at 37 °C. Conditioned medium was collected, and KGFR−HFc was immobilized with heparin−Sepharose, washed three times with buffer, eluted in Laemmli buffer, resolved by SDS−PAGE, and visualized by fluorography.

Mitogenicity Assays. The incorporation of [^{3}H]thymidine into DNA by Balb/MK cells was measured as described previously (*1*).

Binding Assays and Scatchard Analysis. Assays of [^{125}I]KGF and [^{125}I]aFGF binding to the KGFR on intact cells were performed as described previously (*32*). Briefly, confluent cells in multiwell plates were incubated with radiolabeled ligand for 4 h at 4 °C. The cells were washed with ice-cold PBS, and bound radioactivity was extracted with 0.5% SDS and measured by γ counting. Heparin was added in some experiments as indicated. In competition assays, samples contained low levels (1−10 ng) of radiolabeled ligand and several concentrations of competitor; for Scatchard analysis, samples contained various concentrations of radiolabeled ligand in the presence or absence of a 100-fold excess of unlabeled ligand. Estimates of receptor affinity were made using LIGAND software (*37*).

Assays of [^{3}H]heparin binding to KGFR−HFc were performed by incubating KGFR−HFc with [^{3}H]heparin at various concentrations in the presence or absence of a 100-fold excess of unlabeled heparin for 1 h at room temperature. Gammabind G-Sepharose was added for 1 h, and the mixture was collected by centrifugation and washed three times with PBS. The amount of bound [^{3}H]heparin was measured by liquid scintillation counting. Specific binding was defined as the difference between the total binding and counts bound in the presence of excess unlabeled heparin.

Assays of [^{125}I]KGF and [^{125}I]aFGF binding to the KGFR−HFc chimera were similar to the [^{3}H]heparin binding assays described above. Briefly, partially purified KGFR−HFc (20 μg in 200 μL of PBS/0.3% milk) was incubated with varying concentrations of either [^{125}I]KGF (200 000 cpm/ng) or [^{125}I]aFGF (30 000 cpm/ng) for 1 h at room temperature. Bound ligand was immobilized with protein A-Sepharose and washed three times with PBS, and the amount of radioactivity was measured by γ counting. Concentrations of radiolabeled and unlabeled ligand for competitive binding and saturation binding experiments (for Scatchard analysis) were performed essentially as described for assays performed on intact cells. In some experiments, heparin was added during the incubation as noted in the text.

Covalent Affinity Cross-Linking. Covalent affinity cross-linking was performed as described previously (*38*). Briefly, cells were incubated at 4 °C for 3.5 h with 10 ng/mL radiolabeled KGF or aFGF in the presence or absence of heparin (1 μg/mL) in serum-free medium (1 mL) containing 20 mM HEPES (pH 7.3), 0.3% bovine serum albumin (BSA), 0.5 mM $MgSO_4$, and 1 mM $CaCl_2$. Bound ligand was cross-linked with 0.3 mM DSS in ice-cold HEPES-buffered saline (HBS) for 20 min. After quenching, the medium was aspirated, and the cells were washed with ice-

cold HBS and then scraped into HBS containing protease inhibitors. Cells were solubilized in cold lysis buffer containing 10 mM Tris-HCl (pH 7.4), 0.25% NP40, and protease inhibitors. Samples were separated by 7% SDS−PAGE under reducing conditions; the gels were fixed, stained with Coomassie Blue R-250, destained, and dried, and images were generated by autoradiography.

MAP Kinase Activity. MAP kinases (p42 and p44) activated in response to growth factor treatment (100 ng/mL for 10 min at 37 °C) of intact, serum-starved cells were detected by fractionation of SDS cell extracts followed by immunoblotting with an anti-active MAP kinase (New England Biolabs).

Northern Analysis. CHO cells at 90% confluence were serum-deprived (3% FBS) overnight, and then treated with KGF, with or without heparin, for the time periods indicated. Total RNA (15 μg) was extracted using RNA STAT-60 (TEL-TEST "B", Inc., Friendswood, TX), separated on 1% formaldehyde/agarose gels, blotted onto Nytran membranes (Schleicher-Schuell, Keene, NH), and hybridized in Hybrisol I (Oncor, Gaithersburg, MD) at 42 °C with rat *c-fos* (kindly provided by T. Curran) or cyclophilin cDNA (kindly provided by P. E. Danielson) that had been ^{32}P-labeled by random priming (*39*). Membranes were washed using 0.1× SSPE [150 mM NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA (pH 7.4)] with 0.5% SDS at 50 °C. Bound radioactivity was visualized using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Heparin−TSK Affinity Chromatography. The degree of retention of KGF, purified KGFR−HFc, and purified HFc by heparin−TSK was measured using a Heparin-5PW column (7.5 mm inside diameter × 7.5 cm, flow rate of 1 mL/min; TosoHaas) on a Waters 600 HPLC system. The column was equilibrated in PBS (pH 7.4), and samples were eluted with a linear gradient of NaCl from 0.15 to 1.0 M over the course of 1 h. Elution was monitored by UV absorbance at 200−300 nm using a Waters 996 photodiode array detector. The protein elution position was confirmed by collecting 1 min fractions, aspirating these through PVDF membrane using a dot blot apparatus, and immunoblotting with anti-HFc. Retention times listed in Table 1 represent the maximum eluted absorbance at 280 nm that coincided with positive immunostaining.

RESULTS

Heparin Differentially Modulates KGF− and aFGF−KGFR Interactions. In Balb/MK keratinocytes, which express cell-surface heparan sulfate proteoglycan, added soluble heparin is not required for KGF binding or mitogenic signaling (*1*). In fact, added heparin inhibits KGF mitogenic signaling, but enhances signaling by aFGF (Figure 1). Thus, although both ligands bind HSPG and compete for binding to the same receptor, heparin appears to modulate the biological activity of these two ligands differently.

To further investigate the differences between KGF and aFGF with regard to the role of HSPG in ligand binding and signaling, and to determine whether cell-surface HSPG facilitated KGF action, we established stable ectopic KGFR expression in wild type Chinese hamster ovary (CHO) cells, as well as in mutant CHO cells defective in proteoglycan chain initiation (CHO/745). We then compared receptor

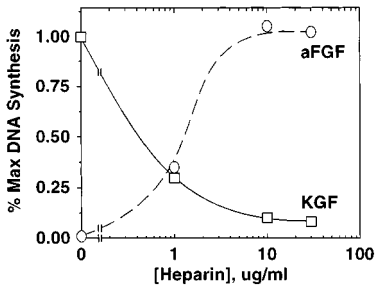

FIGURE 1: Differential effects of added soluble heparin on KGF (□)- and aFGF (○)-stimulated DNA synthesis in Balb/MK keratinocytes. The extent of [^{3}H]thymidine incorporation into DNA, measured as described in Experimental Procedures, is expressed as a percentage of the maximum for KGF (2 ng/mL) or aFGF (10 ng/mL). Values are the mean from triplicate wells, and are representative at least three separate experiments.

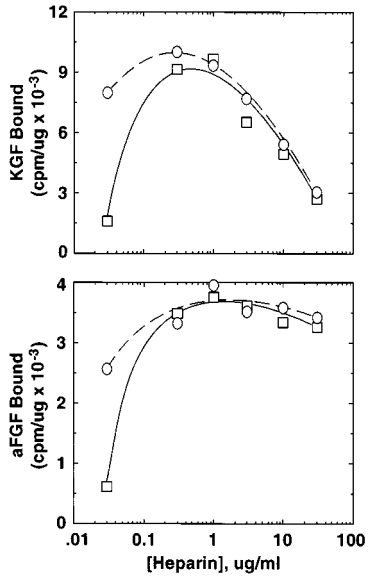

FIGURE 2: Modulation of [^{125}I]KGF (upper panel) and [^{125}I]aFGF (lower panel) binding to wild type CHO (○) and CHO/745 (□) KGFR transfectants by added soluble heparin. Specific binding over a range of heparin concentrations was performed as described in Experimental Procedures. Values shown are the mean of triplicate samples corrected for protein amount; standard errors are smaller than the symbol size. The results are representative of three separate experiments.

binding by KGF and aFGF over a range of heparin concentrations (Figure 2). Binding studies with KGF and aFGF on CHO/745 KGFR transfectants revealed that for both ligands, low concentrations of added heparin (0.1−1.0 μg/mL) dramatically enhanced receptor binding. In contrast, substantial ligand binding was observed for wild type CHO KGFR transfectants in the absence of added heparin, although binding was moderately enhanced by low heparin concentrations. Higher concentrations (3−30 μg/mL) of added heparin affected KGF and aFGF binding differently in both CHO KGFR transfectants. Consistent with our observations of biological activity in Balb/MK cells, increasing concentrations of added heparin had little effect on aFGF binding, but almost completely abolished KGF binding (Figure 2). Identical experiments performed using NIH/3T3 KGFR transfectants yielded results similar to those obtained using the wild type CHO KGFR transfectants, while experiments performed using 32D cell KGFR transfectants, which also lack cell-surface HSPG, yielded results similar to those

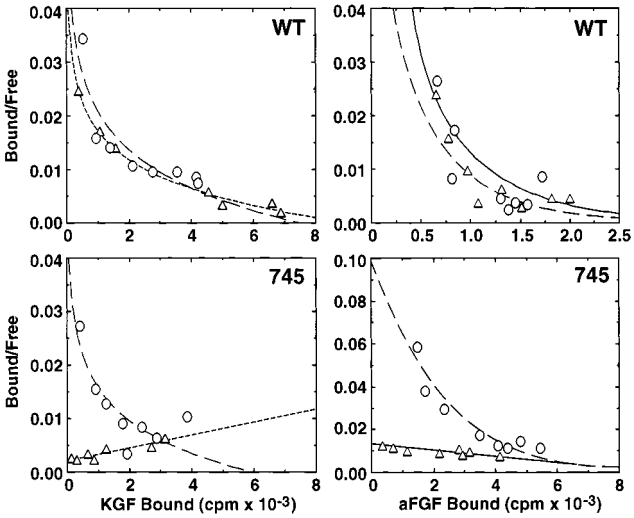

FIGURE 3: Effects of added soluble heparin on the affinity of [^{125}I]KGF (left panels) and [^{125}I]aFGF (right panels) binding to wild type CHO (upper panels) and CHO/745 (lower panels) KGFR transfectants. Shown are Scatchard analysis of samples in the absence (△) or presence (○) of heparin (1 μg/mL). Specific binding values from triplicate samples were corrected for protein amount; the results shown are representative of at least three separate experiments.

obtained with CHO/745 KGFR transfectants (data not shown).

Heparin Enhances Ligand Binding and Signaling in Cells Lacking HSPG. We investigated the mechanism by which heparin modulated ligand−KGFR interaction by Scatchard analysis of saturation binding experiments on the CHO KGFR transfectants performed in the absence and presence of heparin (Figure 3). The apparent affinity of ligand binding to wild type CHO KGFR transfectants was similar in the absence or presence of heparin (1 μg/mL), and modestly enhanced high-affinity binding by aFGF (Figure 3, upper panels). In CHO/745 transfectants, the apparent affinity of ligand binding increased dramatically with added heparin (Figure 3, lower panels).

Covalent affinity cross-linking experiments confirmed that ligand binding to the high-affinity KGFR was facilitated in CHO/745 transfectants by low concentrations (1 μg/mL) of soluble heparin (Figure 4). The major affinity-labeled complex (approximately 145 kDa) observed for both KGF and aFGF is consistent with a ligand:receptor stoichiometry of 1:1, although for KGF, but not aFGF, there appears to be substantially higher-molecular weight complexes that are not well resolved (Figure 4). Figure 4 also shows that the same low concentrations of added heparin (1 μg/mL) had no apparent affect on ligand−KGFR cross-linking on wild type CHO cells, consistent with the binding data shown in Figures 2 and 3.

The effects of heparin on biological signaling via the KGFR in the CHO transfectants were examined by immunoblot analysis of active MAP kinase, and by northern analysis of *c-fos* expression (Figure 5). While p42 and p44 MAP kinases in CHO/745 transfectants were not activated by KGF in the absence of heparin, they were dramatically activated by KGF added in the presence of 1 or 10 μg/mL soluble heparin (Figure 5A). In contrast, added heparin was not required to observe MAP kinase activation by KGF in wild type CHO KGFR transfectants, and activity was modestly

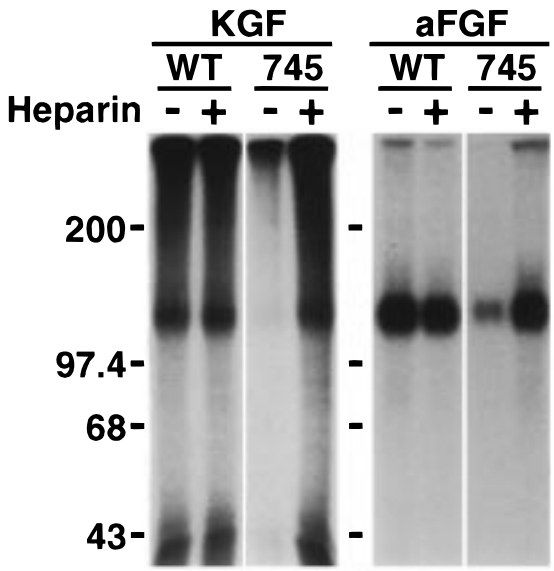

FIGURE 4: Effects of added soluble heparin (1 μg/mL) on the covalent affinity cross-linking of [^{125}I]KGF (left panel) and [^{125}I]aFGF (right panel) to wild type CHO (left two lanes) and CHO/745 (right two lanes) KGFR transfectants. Autoradiograms from dried 7% SDS−PAGE gels were prepared as described in Experimental Procedures. Results shown are representative of at least three separate experiments.

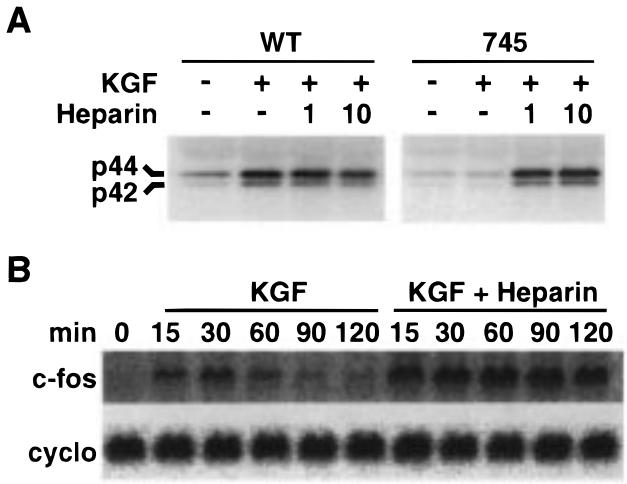

FIGURE 5: Effects of added soluble heparin on KGF signaling in intact CHO cells. (A) Effects of heparin on MAP kinases (p42 and p44) activated in response to KGF treatment of wild type CHO (WT; left panel) and CHO/745 (right panel) KGFR transfectants. Intact cells were treated with KGF (100 ng/mL for 10 min at 37 °C), lysed, and subjected to SDS−PAGE, immunoblotting with an anti-active MAP kinase antibody, and chemiluminescent detection. (B) Effects of heparin on KGF-stimulated *c-fos* induction in CHO/745 KGFR transfectants. Cells were serum-deprived overnight and then treated with KGF without (left half) or with heparin (right half) for the time periods indicated. Total RNA was extracted, separated on formaldehyde/agarose gels, blotted onto Nytran membranes, hybridized with ^{32}P-labeled rat *c-fos* (upper panel) or cyclophilin (lower panel) cDNA, and visualized using a PhosphorImager.

attenuated by heparin at 10 μg/mL. Consistent with the pattern observed for MAP kinase, soluble heparin had no effect on KGF-stimulated *c-fos* induction in wild type CHO KGFR transfectants (data not shown), while KGF-stimulated *c-fos* induction in CHO/745 transfectants was dramatically enhanced in the presence of heparin at 1 μg/mL (Figure 5B). Soluble heparin did not elicit biological responses in either

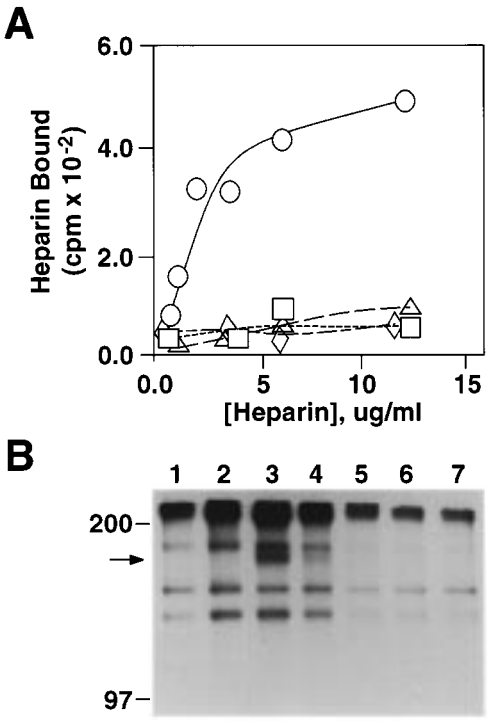

FIGURE 6: [^{3}H]Heparin and heparin−Sepharose binding to KGFR−HFc. (A) [^{3}H]Heparin at the indicated concentrations was incubated with KGFR−HFc (○), HFc (□), control monoclonal antibody MOPC21 (△), or Gammabind G-Sepharose alone (◇) for 1 h. After addition of Gammabind G-Sepharose for 1 h, complexes were captured by centrifugation, and the amount of bound [^{3}H]heparin was measured by scintillation counting. (B) Heparin−Sepharose (lanes 1−3 and 5−7) or Sepharose alone (lane 4) was incubated with metabolically radiolabeled conditioned medium from NIH/3T3 cells (lane 1), NIH/3T3 HFc transfectants (lane 2), or NIH/3T3 KGFR−HFc transfectants (lanes 3−7). The position of the KGFR−HFc protein is indicated by an arrow. Heparin−Sepharose binding by radiolabeled KGFR−HFc was competed with soluble heparin at 70 (lane 5), 250 (lane 6), and 500 μM (lane 7). Captured proteins were analyzed by SDS−PAGE and fluorography.

cell line when added alone, but significantly blocked KGF signaling in both cell lines at concentrations of ≥20 μg/mL (data not shown).

Heparin and Heparan Sulfate Binding by the KGFR. The biphasic effect of added heparin on KGF binding and signaling in intact cells suggested that HSPG may modulate KGF−KGFR interactions by binding to two distinct sites: a higher-affinity site that facilitates ligand−receptor interaction and a lower-affinity inhibitory site. Cell-surface HSPG may be sufficient to facilitate ligand binding and signaling on most cell types through occupancy limited to the higher-affinity site, while on CHO/745 KGFR transfectants, this site must be filled with added heparin. The lower-affinity site which specifically inhibits KGF−KGFR interaction is observed at higher heparin concentrations. Kan and co-workers showed that FGFR-1 binds heparin, and that the heparin binding region of the receptor is important for biological signaling (*31*). To investigate heparin and heparan sulfate binding by the KGFR, and to further characterize ligand−heparin−receptor interaction in the absence of other cell-surface molecules, we used a soluble KGFR−immunoglobulin heavy chain (HFc) chimera described previously (*36*).

As shown in Figure 6A, the binding of [^{3}H]heparin to KGFR−HFc was saturable in the range of 5−10 μg/mL, and

Table 1: Heparin−TSK Affinity Chromatography (HTAC) of Purified KGFR−HFc Chimeras Produced in either Wild Type CHO or CHO/745 Cells Performed As Described in Experimental Procedures

sample	cell type	HTAC retention time (min)
KGFR−HFc	CHO	30
KGFR−HFc	CHO/745	30
KGF	N/A[a]	48
IgG−Fc	CHO	3

[a] N/A indicates not applicable; see the Results for details.

Table 2: K_D Values[a] (Picomolar) for KGF−KGFR Interaction, Estimated by Scatchard Analysis of the Extent of [^{125}I]KGF Binding to Soluble KGFR−HFc Chimeras in the Presence of Heparin

cell type	0 μg/mL heparin	3 μg/mL heparin	300 μg/mL heparin
CHO/WT	375	371	866
CHO/745	242	227	756

[a] Each value is representative of at least three separate experiments.

Scatchard analysis of these data yielded affinity estimates of approximately 200 nM. The heparin binding site was contained in the KGFR portion of the chimera, since [^{3}H]heparin failed to bind the Fc portion of IgG, control monoclonal antibody MOPC21, or Gammabind G-Sepharose alone (Figure 6A). We also tested whether the KGFR−HFc interacted with heparin−Sepharose. KGFR−HFc was readily immobilized by heparin−Sepharose, but not by Sepharose alone (Figure 6B). Under the same conditions, heparin−Sepharose failed to bind the HFc portion of IgG. Figure 6B also shows that KGFR−HFc immobilized by heparin−Sepharose could be eluted with added soluble heparin.

A systematic comparison of the heparin binding properties of the KGFR−HFc, HFc, and KGF was performed by HPLC of heparin−TSK-immobilized proteins using a linear gradient of increasing salt concentration. As shown in Table 1, purified KGFR−HFc chimeras expressed by either wild type CHO or CHO/745 cells were retained by immobilized heparin and were eluted 30 min after the start of the linear NaCl gradient, at a concentration of 504 mM NaCl. IgG heavy chain Fc alone was not retained by heparin−TSK, and KGF was retained and eluted after 48 min, at 760 mM NaCl (Table 1). All of these data suggest that the KGFR extracellular domain binds heparin and heparan sulfate specifically, but with an affinity lower than that of KGF itself.

Heparin-Independent Ligand Binding by a Soluble KGFR−HFc Chimera. To avoid HSPG contamination of the soluble KGFR−HFc chimera, the construct was expressed in HSPG-deficient CHO/745 cells. For comparison, the ligand binding properties of the receptor construct expressed by wild type CHO cells were studied in parallel. Table 2 shows KGF binding affinities for KGFR−HFc produced by each cell type, as estimated by Scatchard analysis of [^{125}I]KGF saturation binding experiments performed in the absence or presence of low (3 μg/mL) and high (300 μg/mL) concentrations of soluble heparin. The relatively minor difference in the KGF binding affinity of KGFR−HFc produced by wild type versus mutant CHO cells (375 vs 242 pM, respectively) suggests that any HSPG that may have contaminated the wild type preparation did not enhance KGF binding. Similarly, when ligand binding to NIH/3T3-expressed KGFR−HFc was

analyzed before and after high-salt extraction of the chimera to remove contaminating HSPG, both KGF and aFGF binding affinities were unchanged (data not shown). Consistent with the effects of added heparin on KGF activity on target cells, high concentrations of heparin diminished the affinity of KGF−KGFR interaction. Most interestingly, high-affinity KGF binding was observed in the absence of HSPG or added heparin, and a moderate amount of added heparin had no apparent affect on ligand−receptor interaction. Thus, unlike the KGFR expressed on the surface of target cells, neither heparin nor HSPG is required to reconstitute high-affinity KGF−KGFR interactions in a defined, soluble binding system.

DISCUSSION

Early studies of KGF noted its strong HSPG binding, but also that it diverged biologically from other well-studied FGFs in that added soluble heparin potently inhibited KGF-stimulated cell proliferation (*1*, *32*, *33*). These initial observations prompted us to systematically characterize KGF−KGFR−HSPG interactions. The distinct mitogenic properties of KGF and aFGF on Balb/MK keratinocytes in the presence of added heparin indicate that two different FGFs with overlapping binding sites on the same receptor can have different HSPG requirements. Because HSPG is abundantly expressed on the surface of Balb/MK cells, these experiments did not rule out the possibility that a certain amount of endogenous HSPG was critical for KGF binding and signaling, as shown previously for aFGF and bFGF acting through FGFR-1 (*16*−*19*, *40*−*42*). Our effort to explore that possibility using HSPG-negative CHO/745 KGFR transfectants demonstrated that KGF−KGFR interaction was facilitated by added heparin in that setting, consistent with a previous report by Jang and co-workers (*43*). Similar to results reported by Roghani et al. (*16*) for bFGF, Scatchard analysis of KGF and aFGF binding to CHO/745 cells revealed that added heparin increased the apparent affinity of ligand−receptor interaction.

Consistent with predictions based on the Balb/MK model, concentrations of added heparin above an optimal threshold of approximately 1 μg/mL inhibited KGF binding and signaling, but not aFGF binding, in the CHO KGFR transfectants. The biphasic effect of heparin on KGF−KGFR interaction in CHO/745 cells suggests that soluble heparin may bind to two distinct sites within the KGF−KGFR complex. The higher-affinity site, most apparent in HSPG-negative cells, may be occupied by added heparin in these systems, and by endogenous HSPG in most other KGF target cells. Occupancy of this site apparently facilitates and/or stabilizes high-affinity ligand−KGFR interaction. The lower-affinity heparin binding site, i.e., the site filled at higher heparin concentrations, apparently inhibits KGF, but not aFGF, binding and signaling through the KGFR in any setting.

While the known HSPG−KGF interaction could account for one of the two putative HSPG binding sites in this model, the physical location of the other site was unclear. The reported interaction between HSPG and FGFR-1 (*31*) prompted us to test whether the second site resided on the KGFR. We also compared the relative heparin binding affinities of KGF and its receptor using a soluble KGFR−HFc chimera whose ligand binding properties are comparable to those of surface-bound KGFR expressed in epithelial target cells (*36*). The soluble KGFR−HFc chimera was specifically immobilized by heparin−Sepharose and heparin−TSK, and elution at 0.5 M NaCl suggests that heparin−KGFR interaction is similar in strength to heparin−FGFR1 interaction (*31*), but significantly weaker than heparin−KGF interaction. Scatchard analysis of [^{3}H]heparin−KGFR binding indicated an affinity constant of ∼200 nM, substantially weaker than those reported for aFGF− or bFGF−heparin interaction (∼2 and ∼60 nM, respectively; *44*−*46*) or estimated for KGF (∼10 nM; D. P. Bottaro, unpublished observations). Thus, our results are consistent with a model in which ligand−HSPG interaction facilitates signaling through the KGFR, while KGFR−HSPG interaction has ligand-specific consequences on signaling: permissive for aFGF but inhibitory for KGF.

While the HSPG binding site on the KGFR has not been precisely identified, it may reside in a portion of the amino terminus of the second IgG-like domain corresponding to the HSPG binding site on FGFR-1 identified by Kan et al. (*31*). That stretch of 18 amino acids rich in basic residues (NH_2-KMEKKLHAVPAAKTVVKFK-COOH) differs from the corresponding KGFR sequence by only one nonconservative substitution (NH_2-KMEKRLHAVPAANTVVKFR-COOH). In support of this idea, a chimeric molecule containing only the second IgG-like domain of the KGFR was retained by heparin−Sepharose and eluted at the same NaCl concentration as the KGFR−HFc chimera used here (H.-G. Cheon and W. J. LaRochelle, unpublished observations).

Scatchard analysis of KGF−KGFR−HFc interaction yielded similar estimated affinities (∼300 pM) in the absence or presence of low concentrations of soluble heparin. Thus, unlike cell-surface KGFR, high-affinity KGF−KGFR interaction in this soluble binding system did not exhibit HSPG dependence. High concentrations of soluble heparin diminished the affinity of the KGF−KGFR−HFc interaction, consistent with the attenuation of KGF binding and biological activity observed when high concentrations of heparin were added to intact cells. These results suggest that high-affinity ligand binding is an intrinsic property of the receptor, and that the fundamental difference between the HSPG dependence of ligand binding to cell-surface receptor and soluble receptor may be due to other molecule(s) present on cell surfaces.

The existence of a cell-surface molecule that masks or otherwise negatively modulates high-affinity KGF−KGFR interaction, glypican, has been demonstrated previously (*47*). However, it remains to be determined whether this particular proteoglycan is responsible for the effects reported here. Given the higher affinity of KGF−HSPG interaction relative to KGFR−HSPG interaction, HSPG−KGF interaction may initially promote KGF−KGFR binding on the cell surface by disrupting the interaction between the KGFR and a negative proteoglycan modulator, such as glypican. In view of the importance of FGF−HSPG complex-induced receptor dimerization for signaling by FGFR-1 (*30*), HSPG-mediated KGF dimerization (or oligomerization) may, in turn, promote KGFR dimerization and subsequent downstream signaling.

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