Potentiation and Inhibition of bFGF Binding by Heparin: A Model for Regulation of Cellular Response[†]

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ABSTRACT: Basic fibroblast growth factor (bFGF) binds to cell surface tyrosine kinase receptor proteins and to heparan sulfate proteoglycans. The interaction of bFGF with heparan sulfate on the cell surface has been demonstrated to impact receptor binding and biological activity. bFGF receptor binding affinity is reduced on cells that do not express heparan sulfate. The addition of soluble heparin or heparan sulfate has been demonstrated to rescue the bFGF receptor binding affinity on heparan sulfate deficient cells yet has also been shown to inhibit binding under some conditions. While the chemical requirements of the heparin−bFGF−receptor interactions have been studied in detail, the possibility that heparin enhances bFGF binding in part by physically associating with the cell surface has not been fully evaluated. In the study presented here, we have investigated the possibility that heparin binding to the cell surface might play a role in modulating bFGF receptor binding and activity. Balb/c3T3 cells were treated with various concentrations of sodium chlorate, so as to express a range of endogenous heparan sulfate sites, and [^{125}I]bFGF binding was assessed in the presence of a range of heparin concentrations. Low concentrations of heparin (0.1−30 nM) enhanced bFGF receptor binding to an extent that was inversely proportional to the amount of endogenous heparan sulfate sites present. At high concentrations (10 μM), heparin inhibited bFGF receptor binding in cells under all conditions. The ability of heparin to stimulate and inhibit bFGF−receptor binding correlated with altered bFGF-stimulated tyrosine kinase activity and cell proliferation. Under control and chlorate-treated conditions, [^{125}I]heparin was observed to bind with a high affinity to a large number of binding sites on the cells (K_d = 57 and 50 nM with 3.5 × 10^6 and 3.6 × 10^6 sites/cell for control and chlorate-treated cells, respectively). A mathematical model of this process revealed that the dual functions of heparin in bFGF binding were accurately represented by heparin cell binding-mediated stimulation and soluble heparin-mediated inhibition of bFGF receptor binding.

Basic fibroblast growth factor (bFGF)[1] is a member of a family of growth factors that affect the growth and differentiation of a large number of cell types (*1*). These growth factors have structural and sequence similarities, and in addition, they all bind to heparin with high affinity (*2*). The ability to bind heparin greatly facilitated the purification of these proteins, and in recent years, it has been shown to be a reflection of physiologically important interactions with heparan sulfate proteoglycans on cell surfaces and within extracellular matrixes (*2−4*). For example, the ability of bFGF to bind to its cell surface tyrosine kinase receptors is dramatically affected by the presence of heparan sulfate. The extent of bFGF binding to its receptors is significantly reduced in cells that either do not express heparan sulfate, have been treated with the sulfation inhibitor chlorate, or have been subjected to heparinase digestion (*5−7*). Furthermore, the reduced level of receptor binding in the absence of heparan sulfate translates to decreased bFGF biological activity (*6*, *8−10*).

The role of heparan sulfate in modulating bFGF receptor binding has been the subject of many studies (see ref *2* for a review). A model that has evolved from these studies suggests that bFGF, its receptor, and a heparan sulfate proteoglycan interact simultaneously to form a high-affinity complex. The heparan sulfate chain could act by binding both bFGF and its receptor, facilitating the bFGF−receptor interaction (*7*, *11*, *12*). In this manner, the cell surface proteoglycan and receptor could each bind bFGF and then come together to form a stable complex. Different forms of the receptor and alterations in heparan sulfate structure might affect bFGF cell binding and cellular response. In addition, localization of heparan sulfate away from the cell surface might result in a heparan sulfate-mediated decrease in the extent of bFGF binding. Indeed, we have previously found that perlecan containing heparan sulfate secreted by endothelial cells is a potent inhibitor of bFGF receptor binding and mitogenesis (*13*, *14*). These opposing activities of heparan sulfate proteoglycans are consistent with reports of both stimulation and inhibition of bFGF binding and activity by exogenous heparin (see ref *2* for a review).

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[1] Abbreviations: bFGF, basic fibroblast growth factor; DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; FGFR, fibroblast growth factor receptors; HSPG, heparan sulfate proteoglycan; MAP kinase, mitogen-activated protein kinase; PBS, phosphate-buffered saline.

The specific heparin and heparan sulfate structures required for interaction with bFGF have been studied in detail (*15*–*20*). The minimum fragment required for binding bFGF is a pentasaccharide (*18*); however, longer fragments are required to enhance bFGF binding to its receptors on cells (*17, 19, 20*). Although most studies have focused on the ability of heparin to rescue bFGF receptor binding on heparan sulfate deficient cells, several have reported inhibition of bFGF receptor binding and activity by soluble heparin (*14, 21*–*23*). The mechanism of inhibition has generally been attributed to the ability of soluble heparin to compete with cell-associated heparan sulfate. However, inhibition of bFGF binding by heparan sulfate has been observed in chlorate-treated cells that do not express significant amounts of sulfated heparan sulfate (*13*), suggesting additional mechanisms for inhibition. One possibility is that the cellular localization of the exogenous heparin has an important impact on bFGF regulatory activity. Heparin has been demonstrated to bind specifically to a number of cell types (*24*–*27*). Thus, it is possible that exogenous heparin can have both stimulatory and inhibitory activities, where heparin binding to cells enhances bFGF–receptor interactions with the remaining soluble heparin acting by binding bFGF in solution, inhibiting its cell surface binding. The net effect would be dependent on the concentration of heparin, the number of heparan sulfate sites on the target cell, and the affinity and number of binding sites for heparin on the target cells.

In the study presented here, we have correlated the effects of exogenous heparin on bFGF receptor binding in Balb/c3T3 cells to the level of endogenous heparan sulfate in these cells, and to the ability of heparin to bind to the cell surface. We further demonstrate that the ability of heparin to stimulate and inhibit bFGF–receptor binding correlated with altered bFGF-stimulated tyrosine kinase activity and cell proliferation. A mathematical model of this process revealed that the dual functions of heparin in bFGF binding were accurately represented by heparin cell binding-mediated stimulation and soluble heparin-mediated inhibition of bFGF binding.

MATERIALS AND METHODS

Materials. bFGF (human recombinant, 18 kDa) was from Scios-Nova (Mountain View, CA). [³H]Thymidine, [³H]-heparin, [³⁵S]sulfate, and [¹²⁵I]Bolton–Hunter reagent were obtained from DuPont-NEN (Boston, MA). [¹²⁵I]bFGF was prepared using a modification of the Bolton–Hunter method (*7*). Sodium chlorate (NaClO₃) was from Fluka (Ronkonkoma, NY). Phosphate-buffered saline (PBS) was from Gibco (Grand Island, NY). Horseradish peroxidase-linked anti-mouse IgG (from sheep) whole antibody was from Amersham (Arlington Heights, IL). Anti-active mitogen-activated protein kinase (MAP kinase) was from Promega (Madison, WI). Anti-phosphotyrosine monoclonal antibody (PY20) was from Transduction Laboratories (Lexington, KY). Heparinase I (EC 4.2.2.7) purified from *Flavobacterium heparinum* was a generous gift of R. Sasisekharan at the Massachusetts Institute of Technology (Cambridge, MA) (*28*). Heparin, chondroitin sulfate, cetylpyridinium chloride, HRP-linked anti-rabbit IgG, and acid phosphatase substrate were from Sigma (St. Louis, MO). Bovine serum albumin was from Intergen (Newark, NJ).

Cell Culture. Balb/c3T3 fibroblasts were from the American Type Culture Collection. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco), supplemented with penicillin (100 units/mL), streptomycin (100 μg/mL) (Gibco), glutamine (2 mM), and 10% calf serum (Hyclone) (*7, 29*). Cells were passaged every 3–5 days while subconfluent by treatment with 0.05% trypsin (Gibco) and with 0.53 mM EDTA, and fresh cells were thawed every 2 months. Cell numbers were measured by a Coulter counter after trypsinization and/or by acid phosphatase activity (*30*). All experiments were conducted using confluent Balb/c3T3 cells plated at a density of 39 000 cells/cm².

Acid Phosphatase Assay. Cells were washed once with PBS. Acid phosphatase buffer was added [0.1 M NaCH₃-COO (pH 5.5), 0.1% Triton X-100, and 0.38 mg/mL *p*-nitrophenyl phosphate disodium], and the cells were incubated at 37 °C for 45 min. The reaction was quenched with 50 μL of 1 N NaOH per well. The sample in PBS was read in a spectrophotometer at an absorbance of 410 or 440 nM (*30*).

Heparin Iodination. Iodinated heparin was a generous gift of J. D. San Antonio (Thomas Jefferson University, Philadelphia, PA) and was prepared as described previously (*31*). Briefly, tyramine was added to the reducing end of heparin and then iodinated using the chloramine-T method. This method iodinates the positions ortho to the hydroxyl group on tyrosine. The heparin was passed over a G-25 column to separate free ¹²⁵I from heparin. The major midsize peak of radiolabeled heparin with an average molecular mass of ~18000 Da was collected and used for binding studies.

Chlorate Treatment. Cells were plated at a density of 39 000 cells/cm² in DMEM with 10% calf serum, penicillin, streptomycin, and glutamine at 37 °C for all experiments. After 24 h, medium was changed to DMEM with 2% dialyzed calf serum (Sigma) and glutamine (2 mM) with or without chlorate at the indicated concentrations. Cells were incubated for 72 h at 37 °C.

[³⁵S]Sulfate Incorporation. [³⁵S]Sulfate (100 μCi/mL final concentration) was added to cells with and without chlorate and incubated for the indicated times. Medium was collected; 2 mM PMSF was added, and samples were stored on ice. Cells were washed once with PBS and then extracted with cold scraping buffer [1 M urea, 1 mM DTT, 10 mM Tris-HCl (pH 7.4), 10 mM EDTA, and 2 mM PMSF] with a Teflon cell lifter. Samples were stored on ice for 10 min and then centrifuged for 10 min at 12000*g*. Supernatant (Medium) was saved and stored on ice. The cell supernatant (ECM) was stored on ice. The cell pellet was dissolved in TUT [10 mM Tris-HCl (pH 8.0), 8 M urea, 0.1% Triton X-100, 1 mM sodium sulfate, and 2 mM PMSF], stored on ice for 10 min, and centrifuged for 10 min at 10000*g*. The supernatant (cell extract) was collected and stored on ice.

Cetylpyridinium Chloride Precipitation. To quantitate the amount of radiolabeled glycosaminoglycans present in cell, ECM, and medium fractions after treatment with heparin, glycosaminoglycans were precipitated using cetylpyridinium chloride, a lipophilic cation which binds to charged groups along the glycosaminoglycans rendering them insoluble (*32*). Cells in 24-well Costar plates were treated with [³⁵S]sulfate with or without 50 mM chlorate for 72 h and separated into medium, extracellular matrix, and cell extract fractions as described previously (*9*). Cells were washed three times in cold binding buffer. Binding buffer (0.5 mL/well) was added, and cells were incubated for 10 min at 4 °C. Heparin was

Table 1: Different bFGF Binding Interactions Are Released by Specific Wash Conditions[a]

	bFGF–HSPG	bFGF–FGFR	bFGF bound to both FGFR and HSPG	heparin bound to bFGF bound to FGFR	bFGF bound to heparin bound to FGFR	bFGF bound to both surface-bound heparin and FGFR	bFGF bound to surface-bound (not FGFR) heparin
2 M NaCl in 20 mM HEPES (pH 7.4)	X				X		X
20 mM sodium acetate (pH 4.0)		X		X			
2 M NaCl in 20 mM sodium acetate (pH 4.0)	X	X	X	X	X	X	X

[a] For low pH, bFGF bound to its receptors only would be released by low pH. If bound to its receptor while also bound to heparin, that is, in solution, bFGF would be released with a low-pH wash. For high salt, bFGF bound to a cell surface-bound heparin would be released by high salt. bFGF bound to a receptor-bound heparin, but not to its receptor, would also be released by high-salt buffers. For low pH with high salt, a low-pH wash with high salt would release bFGF from any interactions at the cell surface since the receptor binding is pH-sensitive and the heparin or HSPG binding is salt-sensitive. The information in this table was generated on the basis of previously published data (5–7, 9, 11, 33).

added at varying concentrations, and the cells were incubated at 4 °C for 1 h. Medium, ECM, and cell extract fractions were collected as described above. Heparin (20 µL of a 10 mg/mL solution) was added to 80 µL of each sample as a carrier, and then 20 µL of PBS and 80 µL of 5% cetylpyridinium chloride hydrate were added. Samples were vortexed, incubated at 37 °C for 1 h, and microfuged for 10 min at 10000g. The pellet was resuspended in 1% cetylpyridinium chloride and microfuged for 10 min at 10000g. The resulting pellet was dissolved in 100 µL of 0.5 M sodium acetate, and samples were counted in a Packard A2010 scintillation counter.

bFGF Binding. bFGF binding is complex, involving binding to receptors, HSPG, and complexes of both. To analyze these interactions, a series of binding studies were performed with Balb/c3T3 cells. Equilibrium binding of [^{125}I]-bFGF was conducted with confluent Balb/c3T3 cells (7, 9). Cells in 24-well plates (Costar, Cambridge, MA) were washed once with binding buffer (DMEM, 25 mM HEPES, and 0.05% gelatin) at 4 °C. Fresh binding buffer was added (0.5 mL/well), and cells were incubated at 4 °C for 10 min. [^{125}I]bFGF was added at the indicated concentrations, and cells were incubated at 4 °C for 2.5 h. At the end of the binding period, cells were placed on ice and washed three times with ice-cold binding buffer. HSPG-bound [^{125}I]bFGF was removed with a quick wash (~5 s) using 2 M NaCl in 20 mM HEPES (pH 7.4) followed by a wash with PBS (33). Cell surface receptor-bound [^{125}I]bFGF was subsequently extracted with two washes (one 5 min wash and one rapid wash) at room temperature using 2 M NaCl in 20 mM sodium acetate (pH 4.0). The level of nonspecific binding was measured and subtracted from all data (9). Variations in the nature and the order of the washes were conducted to further characterize bFGF binding (Table 1). Bound bFGF was released from cell-associated binding sites using a variety of extraction buffers: (1) 2 M NaCl in 20 mM HEPES (pH 7.4) followed by a PBS wash to remove bFGF bound to HSPG alone, and not bound to receptors; (2) 20 mM sodium acetate and 150 mM NaCl (pH 4.0) followed by an identical wash to remove bFGF bound to receptors alone, and not bound to HSPG; and (3) 2 M NaCl in 20 mM sodium acetate (pH 4.0) followed by an identical wash to remove bFGF bound to either HSPG, receptor, or a combination of HSPG and receptor.

Control experiments were conducted using cells exhaustively treated with heparinase and/or chlorate (receptor binding only) to determine the amount of receptor-bound bFGF that is released by the high-salt wash. Furthermore, experiments were conducted with cell-free extracellular matrix-coated dishes and with very high concentrations of [^{125}I]bFGF, which would predominantly bind to HSPG sites only, to determine that amount of HSPG-bound bFGF that is released in the low-pH, low-salt wash. It was estimated that the 2 M NaCl, pH 7.4 solution extracted >95% of the HSPG-bound and <5% of the receptor-bound bFGF. The low-pH, 150 mM NaCl solution was able to extract >95% of the receptor-bound bFGF. The high-salt, low-pH solution [2 M NaCl and 20 mM sodium acetate (pH 4.0)], when used alone (in the absence of a high-salt prewash), was able to extract amounts of bFGF equal to the sum of a high-salt [2 M NaCl in 20 mM HEPES (pH 7.4)] wash followed by the low-pH, high-salt extraction (7, 9, 33). These variations allowed bFGF that was receptor-bound to be isolated from bFGF that was in a complex with receptor and heparin–HSPG.

Heparin Binding. Studies were conducted to assess the ability of heparin to bind to Balb/c3T3 cells. Equilibrium binding of [^{125}I]heparin was conducted with confluent Balb/c3T3 cells. Cells in 24-well plates (Costar) were washed once with binding buffer (DMEM, 25 mM HEPES, and 0.05% gelatin) at 4 °C. Fresh binding buffer was added (0.5 mL/well), and cells were incubated at 4 °C for 10 min. [^{125}I]-Heparin was added at the indicated concentrations, and cells were incubated at 4 °C for 1 h. At the end of the binding period, cells were placed on ice and washed three times with ice-cold binding buffer. NaOH (0.3 N) was added to extract the bound [^{125}I]heparin by overnight incubation at room temperature. Contents of wells were transferred to tubes and counted in a Packard model 5650 γ counter. Experiments were also conducted using 2 M NaCl in 20 mM HEPES (pH 7.4) with [^{3}H]heparin (DuPont-NEN) to confirm that all counts were salt-releasable. In one particular experiment, high-salt extraction released 3517 ± 63 cpm compared to 3220 ± 124 cpm released with NaOH in control cells. Chlorate-treated cells yielded 3022 ± 328 cpm with high salt and 2499 ± 7 cpm with NaOH. Nonspecific binding was assessed empirically by competition with increasing concentrations of cold heparin until no further reduction of labeled heparin binding was observed. The amount of

nonspecific binding was determined to increase linearly with increased [^{125}I]heparin concentration. The specific binding data were analyzed using a modification of the Scatchard method by fitting the data to the following equation to determine the binding constant and number of binding sites assuming a single class of binding site:

$$[\text{heparin}]_b = \frac{R_T[\text{heparin}]_f}{K_d + [\text{heparin}]_f}$$

where K_d and R_T represent the dissociation constant and the number of binding sites, respectively, and [heparin]$_b$ and [heparin]$_f$ represent the measured concentration of specifically bound and free heparin, respectively (*9*, *34*). Curve fitting was conducted using the Levenberg−Marquardt algorithm with the program KaleidaGraph, version 3.0.4 (Synergy Software, Reading, PA), on a Macintosh Power PC.

Western Blot Analyses of MAP Kinase and Phosphotyrosine. Control and chlorate-treated cells were prepared as described. After bFGF treatment, cells were washed twice in PBS; then 1 mL of HTG (20 mM HEPES, 1% Triton X-100, 10% glycerol, and 1 mM PMSF) was added, and cells were scraped with a cell lifter. Lysate was centrifuged at 10000*g* for 10 min. The supernatants were normalized to total protein, run on polyacrylamide gels, and electrotransferred to Immobilon-N (Millipore Corp., Bedford, MA). For anti-active MAP kinase and anti-phosphotyrosine blots, membranes were blocked overnight in TBS-T (Tris-buffered saline solution and 0.5% Tween 20) with 10% BSA. Membranes were hybridized with either anti-active MAP kinase at a dilution of 1:6000 or anti-phosphotyrosine at a dilution of 1:666 in TBS-T with 2% BSA at 37 °C for 1.5 h. Secondary antibody hybridization was carried out with HRP-linked anti-rabbit IgG (Sigma) at a dilution of 1:12500 for 1.5 h in TBS-T with 2% BSA at 37 °C for anti-active MAP kinase blots, or HRP-linked anti-mouse IgG (from sheep, Amersham) at a dilution of 2:5000 in TBS-T with 2% BSA at 37 °C for 1.5 h for anti-phosphotyrosine blots. Bands were visualized with Renaissance Western blot chemiluminescence reagent (DuPont-NEN) on Amersham hyperfilm.

[^{3}H]Thymidine Incorporation. After treatment for 72 h with or without chlorate, bFGF with or without heparin (concentrations indicated in figures) and [^{3}H]thymidine (1 μCi/mL) were added and the cells incubated for 24−36 h at 37 °C. Cells were washed with PBS, and DNA was precipitated with methanol and trichloroacetic acid (*29*). Total [^{3}H]thymidine incorporation was quantitated by liquid scintillation counting.

Mathematical Modeling Methods. A mathematical model based on previous work (*35*) was developed. The model was based on cells with a homogeneous distribution of FGF receptors, heparan sulfate proteoglycan binding sites, and heparin binding "receptors". The HSPG sites and heparin binding receptors were assumed to be homogeneous. This is a valid first approximation since all the binding parameters used in the model represent averaged values for each binding interaction. The volume of the local environment reflects the cell density and the absolute level of bFGF and heparin available per cell.

The model is composed of a system of ordinary differential equations which describe the kinetics of binding of bFGF to FGFR in the presence and absence of surface HSPG and

heparin (Appendix). Our model reflects the experimental binding conditions at 4 °C where internalization and synthesis are assumed to be negligible. The base model (no heparin) is composed of three differential equations and three algebraic equations reflecting the lack of degradation or synthesis of bFGF, FGFR, and HSPG. The equations reflect association and dissociation of bFGF for the cell surface binding elements (FGFR and HSPG) and coupling to form a high-affinity triad (bFGF−FGFR−HSPG). It should be noted that our association and dissociation constants for bFGF binding are not constants but a function of unbound FGFR and HSPG and that the surface coupling rates are a function of the spatial distance between surface molecules reflecting the density of these compounds. We assumed that there were no preformed HSPG-R complexes and that dissociation of bFGF from a triad led to the release of both an HSPG and an FGFR. The inclusion of heparin leads to the addition of four differential equations to our model. Basic FGF was allowed to bind heparin in solution and when heparin was bound to FGFR (*2*). In addition, binding of bFGF−heparin solution complexes with FGFR was included, but bFGF−FGFR surface complex binding to extracellular heparin was not. Our experimental data indicated no substantial increase in the level of salt-sensitive binding of bFGF in the presence of heparin which allowed us to eliminate bFGF binding to surface heparin−heparin receptor complexes and bFGF−heparin complexes binding to heparin receptors, other than FGFR.

The full set of equations which compose our model are shown in the Appendix with the algebraic equations reflecting the lack of overall synthesis or degradation in the system at 4 °C. The parameter values as well as the initial conditions used in our calculations are listed in Appendix. LSODE, an algorithm for numerical integration of nonlinear ordinary differential equations, was used to solve the equations given the initial conditions corresponding to bFGF addition at time zero (*36*, *37*). The solutions shown in Figure 8 correspond to 2.5 h of bFGF exposure which corresponds to the experimental time period of interest. The coupling constants were obtained as previously published using Gaussian quadratures to solve the diffusion equation related to the probability of interaction between a bFGF−FGFR complex and an unbound HSPG or a bFGF−HSPG complex and an unbound HSPG before dissociation of the bFGF can occur (*35*).

RESULTS

Heparin Inhibits and Stimulates bFGF Receptor Binding. Independent studies have shown that cells depleted of heparan sulfate were able to recover their affinity for bFGF binding when soluble heparin was added to the medium (*5*, *10*, *17*, *19*, *38*, *39*). The aim of this study was to gain insight into the mechanisms of the effects of heparin on bFGF. To investigate the effect of soluble heparin on the binding of bFGF, cells were treated with varying amounts of chlorate to regulate the amount of HSPG binding sites on the surface. This range of chlorate concentrations was demonstrated to decrease the number of bFGF-binding HSPG sites but has no effect on the number of bFGF receptors on these cells (*9*). bFGF−HSPG and bFGF−heparin binding has been shown to be salt-sensitive (>1 M NaCl), while bFGF−receptor binding is sensitive to low pH (pH 4) (*33*). These characteristics were exploited in assessing and classifying
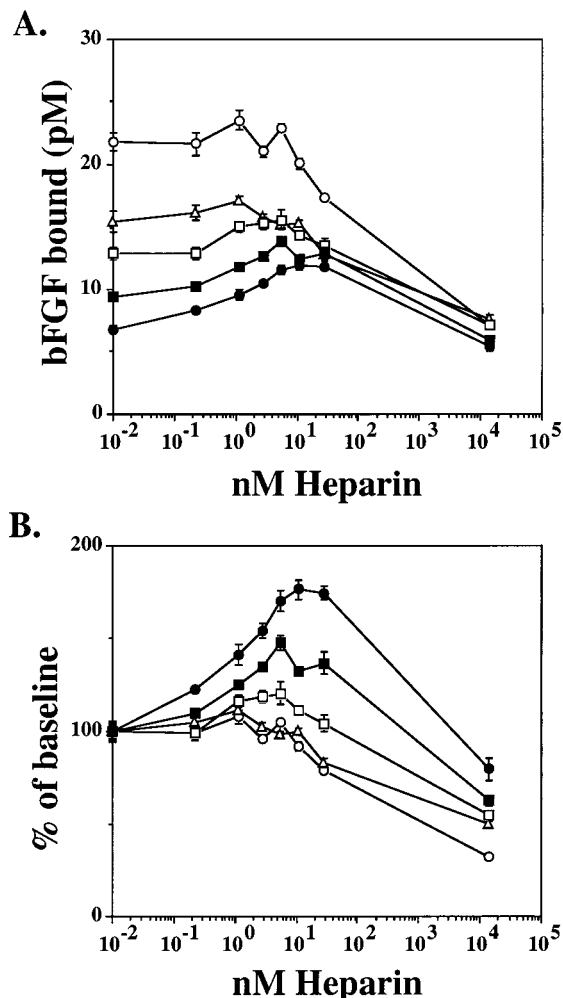
A.

B.

FIGURE 1: Recovery of bFGF binding to its receptor in chlorate-treated cells with the addition of soluble heparin. (A) Balb/c3T3 cells were treated with 0 (○), 10 (△), 15 (□), 25 (■), and 50 mM chlorate (●) for 72 h at 37 °C and incubated at 4 °C for 1 h. [^{125}I]-bFGF was added (0.22 nM, 4 ng/mL), and cells were incubated at 4 °C for an additional 2.5 h. Data represent the amount of receptor-bound bFGF released with a wash of 2 M NaCl and 20 mM sodium acetate (pH 4.0) which followed but does not include a prewash with 2 M NaCl and 20 mM HEPES (pH 7.4). Data points represent the average of triplicate wells, and standard errors of the mean (SE) were determined. (B) Data from panel A represented as a percentage of the baseline level of [^{125}I]bFGF binding at each chlorate concentration when no heparin was added. Control experiments conducted with chondroitin sulfate (0−500 nM) showed neither stimulation nor inhibition of bFGF binding.

different bFGF interactions (Table 1). Increasing heparin concentrations were added to cells, and [^{125}I]bFGF equilibrium binding assays were conducted (Figure 1A). The effect of low heparin concentrations on bFGF binding in control cells was minimal; however, after cells were treated with higher chlorate concentrations, heparin was able to restore binding of bFGF to its receptor. The amount of heparin required to modulate bFGF binding to its optimal level and the ability of bFGF binding to rise above the baseline level at each chlorate concentration were proportional to the degree of inhibition of sulfation. For example, cells that were HSPG-depleted to the greatest extent (50 mM chlorate) required a higher heparin concentration to reach their maximal bFGF binding level but were also able to increase the level of bFGF binding to a greater extent relative to their baseline level (no added heparin) than cells that were less HSPG-depleted
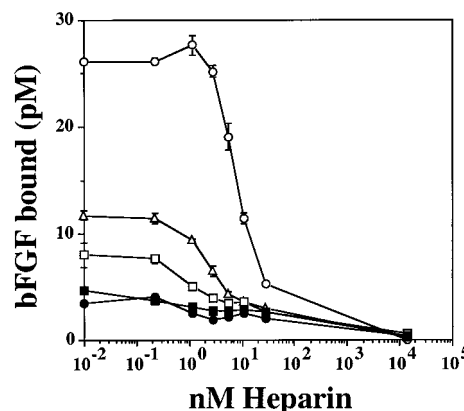

FIGURE 2: Effect of soluble heparin on salt-releasable [^{125}I]bFGF binding in chlorate-treated cells. Balb/c3T3 cells were treated with 0 (○), 10 (△), 15 (□), 25 (■), and 50 mM chlorate (●) for 72 h at 37 °C. Heparin was added at the indicated concentrations, and cells were incubated at 4 °C for 1 h. [^{125}I]bFGF was added (0.22 nM, 4 ng/mL), and cells were incubated at 4 °C for an additional 2.5 h. Data represent the amount of [^{125}I]bFGF released with a wash of 2 M NaCl and 20 mM HEPES (pH 7.4). Data points represent the average of triplicate wells ± SE.

Table 2: Heparin Does Not Cause the Release of Sulfated Proteoglycans from Balb/c3T3 Cells[a]

[heparin] (μM)	medium (cpm)	ECM (cpm)	cell extract (cpm)
0	291	998	2354
5.6×10^{-4} (0.01 μg/mL)	235	845	3678
5.6×10^{-3} (0.1 μg/mL)	332	437	2439
1.12 (20 μg/mL)	386	1060	2549

[a] Balb/c3T3 cells were radiolabeled with ^{35}SO$_4$ (100 μCi/mL) for 72 h at 37 °C and then treated with the indicated concentration of heparin for 1 h at 4 °C. The treatment buffer was collected, and the ECM and cell layers were extracted. ^{35}S-labeled proteoglycan was quantitated in all fractions by CPC precipitation as described in Materials and Methods. The results are the average of triplicate determinations from one representative experiment.

(Figure 1B). It can also be seen in this experiment that cells under all conditions exhibited a relatively similar decreased level of bFGF binding when exposed to high concentrations of heparin. Since the level of bFGF binding is reduced with high soluble heparin concentrations even in cells treated with 50 mM chlorate, which exhibit no bFGF−HSPG binding, it is likely that high soluble heparin concentrations compete with bFGF binding sites by reducing the availability of bFGF at the cell surface. While heparin had both stimulatory and inhibitory effects on bFGF receptor binding, addition of heparin showed only inhibition of bFGF binding to HSPG sites on control and chlorate-treated cells (Figure 2). Figure 2 represents the fraction of bFGF binding that was removed by high-salt treatment. Under all conditions, heparin either had no effect or inhibited bFGF binding to HSPG.

It has been reported that the addition of soluble heparin to hepatocytes induces the release of cell-associated HSPG (40). If this process occurs with Balb/c3T3 cells, it would complicate the interpretation of results obtained with added soluble heparin. To examine this possibility, soluble heparin was added at concentrations up to 20 μg/mL to cells which had been prelabeled with [^{35}S]sulfate. As shown in Table 2, there was no significant release of [^{35}S]glycosaminoglycans with the addition of heparin up to a concentration of 20 μg/mL.
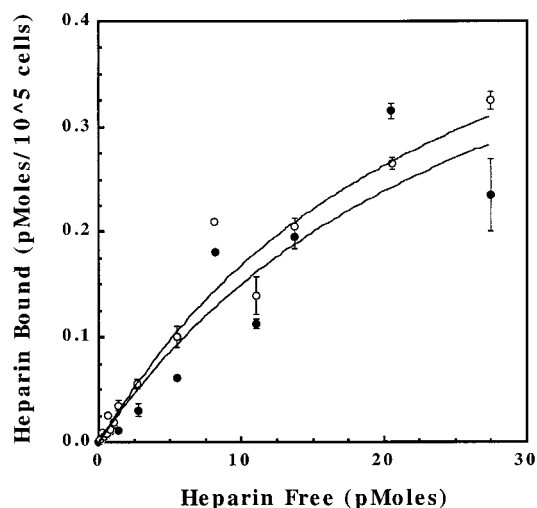
FIGURE 3: Binding of [^{125}I]heparin to Balb/c3T3 cells. Balb/c3T3 cells were treated without (●) or with (○) chlorate (50 mM) at 37 °C for 72 h. A range of [^{125}I]heparin concentrations was added, and the cells were incubated at 4 °C for 1 h. Cells were washed three times with binding buffer, and 0.3 N NaOH was added. Samples were collected and quantitated in a γ counter. The amount of heparin bound was determined and the amount free calculated from the total. The results represent the averages ± SE of triplicate determinations. Line fits were generated using KaleidaGraph, version 3.0.4, as described in Materials and Methods. The upper line represents the fit to the data from chlorate-treated cells (○) and the lower line for that from control cells (●).

Heparin Binds to Control and Chlorate-Treated Cells. One possible mechanism for the dual effects of added heparin on FGF receptor binding could involve a saturable physical association of heparin with the cell surface. Equilibrium binding assays with [^{125}I]heparin were conducted with control and chlorate-treated Balb/c3T3 cells. As shown in Figure 3, heparin bound to cell surfaces with approximately the same apparent dissociation constant under both control and chlorate treatment conditions ($K_d = 57$ vs 50 nM for control and chlorate-treated cells, respectively) and the same number of binding sites (3.5×10^6 vs 3.6×10^6 sites/cell for control and chlorate-treated cells, respectively). Heparin that bound to these sites was releasable with a 2 M NaCl wash alone, suggesting an ionic binding interaction (see Materials and Methods). If the bound heparin were capable of binding bFGF, then it would be predicted that the amount of salt-releasable bFGF binding sites would increase. In the presence of 50 nM heparin, the heparin binding study predicts that there would be ~1.8×10^6 new heparin molecules on the cell surface for bFGF to bind. However, as shown in Figure 2, there is no increase in the level of bFGF binding to heparin or heparan sulfate sites.

The Increased bFGF Binding in the Presence of Heparin Is Released at Low pH. To more fully characterize the binding of bFGF to chlorate-treated cells in the presence of heparin, variations in the extraction of the salt- and acid-sensitive binding sites were performed (Table 1 and Figure 4). A heparin concentration of 5.6 nM (100 ng/mL) was chosen since it resulted in maximal stimulation of bFGF receptor binding in cells treated with 50 mM chlorate. Equilibrium binding of [^{125}I]bFGF was conducted with chlorate-treated cells. When cells were treated first with a low-pH wash, no additional bFGF binding was released in

fractions from heparin-treated cells compared to cells with no heparin treatment (Figure 4, sequence 1). When the cells were first subjected to a prewash with high salt and then extracted with the low-pH, low-salt solution (sequence 2), the additional bound bFGF was released from the heparin-treated cells. These results suggest that the additional bFGF binding observed when heparin was added to chlorate-treated cells was bound to the cell surface in a salt- and pH-sensitive manner. The selective wash analysis further suggests that the additional bFGF binding, when heparin was added to chlorate-treated cells, resulted from the formation of high-affinity ternary complexes that involve the association of bFGF with heparin and its receptor at the cell surface.

Heparin Regulates bFGF-Mediated Activity. Heparin restored binding to high-affinity receptor sites, suggesting that an increased level of binding could translate into an increased level of signaling. The stimulation of DNA synthesis by bFGF was assayed by assessing [^{3}H]thymidine incorporation in the presence and absence of heparin. A higher heparin concentration of 139 nM (2.5 μg/mL), still within the range of heparin concentrations observed to potentiate bFGF binding, was used since the presence of heparin in cell culture over the length of the mitogenesis experiment will result in significant catabolism (*41*). This concentration of heparin had no effect on bFGF stimulation of [^{3}H]thymidine incorporation in control cells (Figure 5A). However, as can be seen in Figure 5B, the addition of heparin to chlorate-treated cells restored the bFGF stimulation of [^{3}H]-thymidine incorporation to control levels, causing approximately a 10-fold shift in the ED_{50}.

One of the first signaling events to take place after bFGF–receptor binding is tyrosine phosphorylation of the receptor, which leads to activation of at least one signaling pathway, the MAP kinase cascade, and ultimately to stimulation of cell proliferation (*1*). Thus, to measure the effects of heparin-mediated bFGF binding throughout the course of the cellular response, two points in the signaling pathway were monitored: overall tyrosine phosphorylation levels and the level of active MAP kinase. These points represent both upstream and downstream events in bFGF-stimulated cellular response. Tyrosine phosphorylation was assayed by anti-phosphotyrosine antibody immunoblotting of cell extracts from control and chlorate-treated cells which had been treated with 0.22 nM (4 ng/mL) bFGF with increasing heparin concentrations (Figure 6). Control cells exhibited an increase in the level of tyrosine phosphorylation when bFGF was added (Figure 6A). The molecular masses of phosphorylated proteins were in the expected range of the FGF receptors (100–130 kDa). As the heparin concentration was increased the signal was mitigated progressively, correlating with the reduction in the level of binding observed with increasingly higher heparin concentrations. Chlorate-treated cells (Figure 6B) exhibited considerably less intense bands when treated with bFGF alone, as would be expected, but exhibited an increase in band intensity when as little as 0.56 nM (10 ng/mL) heparin was added. Higher concentrations of heparin, as in control cells, caused a mitigation of band intensity, indicating that high heparin concentrations inhibit bFGF-induced signaling.

MAP kinase activity was assayed using an anti-active MAP kinase antibody that recognizes the activated forms of
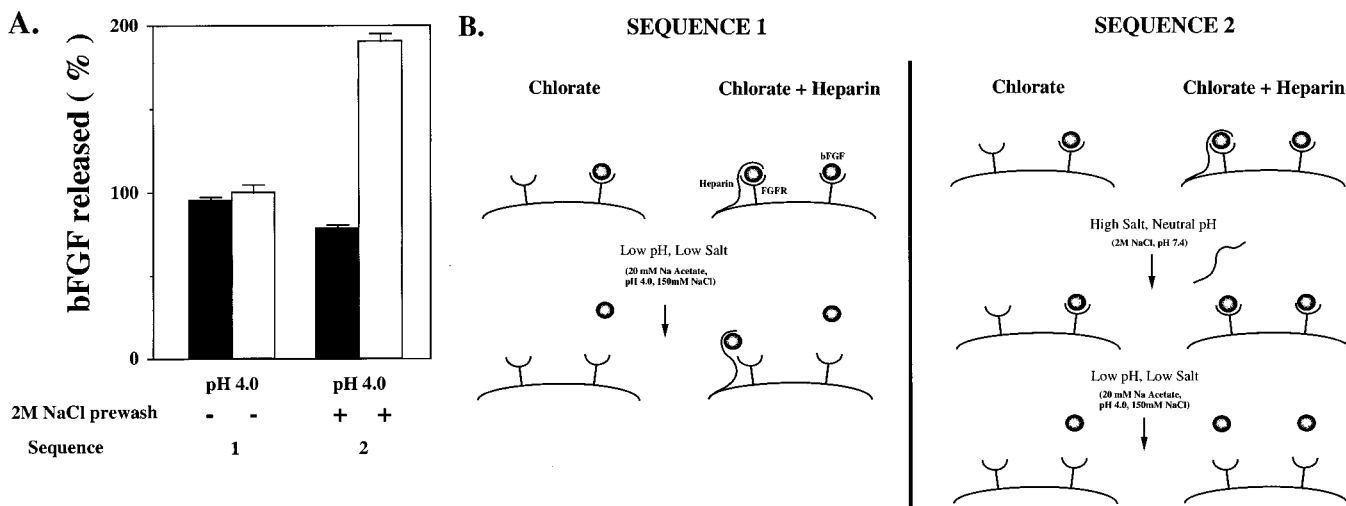
FIGURE 4: Characterization of [^{125}I]bFGF binding to chlorate-treated Balb/c3T3 cells with added heparin. (A) Confluent Balb/c3T3 cells were treated with chlorate (50 mM) for 72 h at 37 °C. Cells were incubated at 4 °C for 1 h with (white symbols) or without (black symbols) heparin (5.6 pM, 100 ng/mL). [^{125}I]bFGF was added (0.22 nM, 4 ng/mL), and cells were incubated at 4 °C for 2.5 h. [^{125}I]bFGF binding to the cells was then evaluated by conducting two extraction sequences. In sequence 1, cells were extracted with 20 mM sodium acetate and 150 mM NaCl (pH 4.0) (low-pH, low-salt treatment). In sequence 2, cells were subjected to a prewash with 2 M NaCl and 20 mM HEPES (pH 7.4) (high-salt, neutral-pH treatment) and then extracted with either 20 mM sodium acetate or 150 mM NaCl (pH 4.0) (low-pH, low-salt treatment). The results represent the amount of bound [^{125}I]bFGF that was released by the low-pH, low-salt wash for each sequence. The data represent the average ± SE of triplicate determinations. All data were normalized to the amount of [^{125}I]bFGF extracted by a single wash with 2 M NaCl (pH 4.0) from non-heparin-treated samples (100%). (B) Schematic of the effects of the various extraction methods in the presence and absence of added heparin. In sequence 1, only bFGF bound to receptor alone and not that bound to cell-associated heparin would be expected to be released by the low-pH, low-salt extraction. In sequence 2, the high-salt, neutral-pH prewash would release heparin from bFGF and cell-associated binding sites (including FGF receptors), such that all cell-bound bFGF would be expected to be released in a subsequent low-pH, low-salt extraction.

extracellular signal-regulated protein kinases 1 and 2. bFGF-treated control cells exhibited high-intensity bands with only a slight reduction in intensity even at high heparin concentrations (Figure 7A). Chlorate-treated cells exhibited a similar response to bFGF with and without heparin, and also exhibited only a slight reduction in intensity at high heparin concentrations (Figure 7B). Thus, the activation of MAP kinase in the presence of added heparin did not correlate with the changes in bFGF–receptor binding. It was possible that maximum activation of MAP kinase was achieved at concentrations of bFGF that were lower than those used in this study (0.22 nM, 4 ng/mL). There could also be differences in the kinetics of the long-term response to bFGF in control and chlorate-treated cells.

Model of Heparin-Mediated Stimulation and Inhibition of bFGF Binding. The ability of heparin to modulate bFGF binding and activity appeared to depend on the endogenous level of HSPG and the concentration of heparin. Heparin was able to both stimulate and inhibit bFGF binding. To test the possibility that the saturable cell surface binding sites for heparin might allow heparin to potentiate bFGF receptor binding while soluble heparin inhibits, we evaluated several mathematical models of this process based on experimentally determined parameters.[2] The parameter values are listed in the Appendix. Computer simulations were run, and theoretical data were generated from a number of models where various possible processes were included or excluded. One model which produced results that were similar to those from

experiments is based on a homogeneous population of cells which are exposed to bFGF at 4 °C for 2.5 h. Under these conditions, we assumed no degradation or production of bFGF, heparin, or any surface molecules during the 2.5 h incubation. Allowing both heparin and heparin–bFGF complexes to bind to cell surface molecules, including the FGFR, led to the prediction of enhanced receptor binding for HSPG-depleted cells (Figure 8A). Furthermore, the theoretical model also predicted the sharp inhibition of the salt-releasable bFGF binding in the presence of heparin (Figure 8B). When simulations were run without allowing heparin to bind to the cell surface; only inhibition of bFGF binding was observed. It should be noted that the level of HSPG binding on chlorate-treated cells increased in cases where enhanced receptor binding was seen; however, the peak binding values were so low (<0.25 pM) that experimental measurements would not be sensitive enough to detect the change.

DISCUSSION

The relationship of HSPG and bFGF has been the subject of many studies (*4–7, 33, 38, 42*). Some of these studies have demonstrated that the function of cell surface HSPG might be replaced, to some degree, by the addition of exogenous HSPG or heparin; however, the exact nature of the mechanism has not been determined. This report extends these studies and further suggests additional interactions. Our data suggest that for heparin to replace the native function of HSPG, it might first need to associate with the cell surface where it can participate in bFGF–receptor interactions. We have determined this by isolating the fractions of [^{125}I]bFGF bound to receptors alone, to HSPG and/or heparin alone, and

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[2] K. E. Forsten, M. Fannon, and M. A. Nugent, Potential Mechanisms for the Regulation of Growth Factor Binding by Heparin (manuscript submitted for publication).
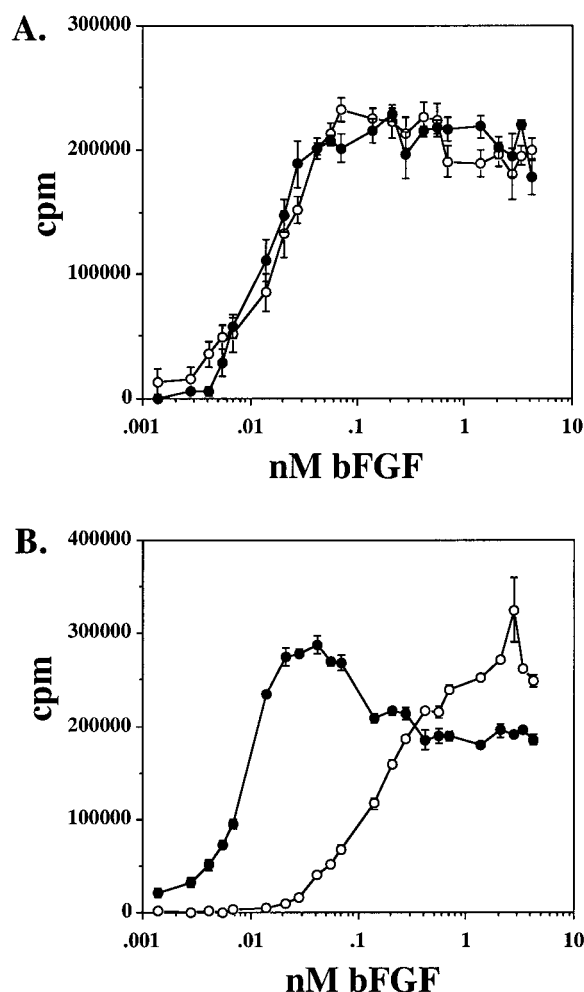
FIGURE 5: Effect of heparin on [³H]thymidine incorporation in control and chlorate-treated cells. Confluent Balb/c3T3 cells were treated without (A) or with (B) chlorate (50 mM) for 72 h at 37 °C. bFGF was added at the indicated concentrations without (○) or with (●) heparin (140 nM, 2.5 μg/mL), and cells were incubated at 37 °C for 24 h. [³H]Thymidine incorporation was assessed by methanol/trichloroacetic acid precipitation of wells (see Materials and Methods). Radioactivity was quantitated in a scintillation counter. The basal level of incorporation of [³H]thymidine in the absence of added bFGF for control and chlorate-treated samples was subtracted from the data in each set. Data points are the average of quadruplicate wells ± SE.

to complexes of receptor with HSPG or heparin (Table 1). This was accomplished by exploiting the ionic and pH-dependent differences of bFGF binding to its tyrosine kinase receptor and to heparan sulfate (*33*). In this way, the effects of added heparin on bFGF binding were assessed on cells that had been treated with variable concentrations of chlorate so as to have a range of endogenous heparan sulfate sites. Low heparin concentrations only potentiated bFGF binding when cells expressed low levels of HSPG. Furthermore, significant amounts of soluble heparin were observed to bind to the cells, suggesting a mechanism whereby heparin might associate with bFGF and its receptor to generate a high-affinity reaction. Once sufficient heparin and endogenous HSPG are present, additional heparin resulted in inhibition of bFGF binding. Several mathematical models for this process were evaluated.[2] Only models that incorporate the interaction of heparin or bFGF–heparin complexes with the cell surface were able to predict the experimental situation.
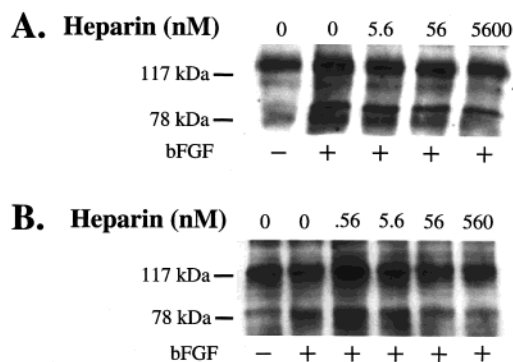

FIGURE 6: Effect of heparin on bFGF-stimulated tyrosine phosphorylation. Confluent Balb/c3T3 cells were treated with or without chlorate (50 mM) for 72 h at 37 °C. Heparin was added at the indicated concentrations, and cells were incubated at 37 °C for 1 h. bFGF was added (0.22 nM, 4 ng/mL), and cells were incubated at 37 °C for 10 min. Cells were extracted, and SDS−PAGE (5%) was performed followed by transfer to membranes. Membranes were hybridized with an anti-phosphotyrosine antibody and visualized with chemiluminescence on film: (A) control and (B) chlorate-treated cells.
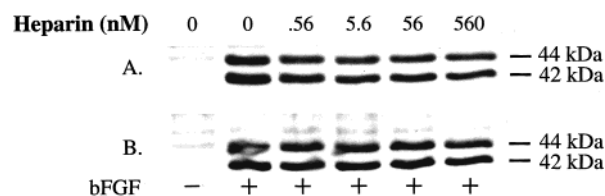

FIGURE 7: Effect of heparin on bFGF-stimulated MAP kinase activation. Confluent Balb/c3T3 cells were treated with or without chlorate (50 mM) for 72 h at 37 °C. Heparin was added at the indicated concentrations, and cells were incubated at 37 °C for 1 h. bFGF was added (0.22 nM, 4 ng/mL), and cells were incubated at 37 °C for 10 min. Cells were extracted, and SDS−PAGE (10%) was performed followed by transfer to membranes. Blots were hybridized with an anti-active MAP kinase antibody and visualized with chemiluminescence on film: (A) control and (B) chlorate-treated cells.

Dimerization was not included in this model since our primary intention was to determine if a simple model could predict the biphasic effects of heparin on bFGF binding. Dimerization may be critical for activity; however, our current data do not allow one to distinguish the effects of bFGF dimers and higher-order oligomers from those of monomeric states, and hence, we did not include this in our model.

Heparin binding to cell surfaces has been reported previously; however, the specific function of this surface-bound fraction in bFGF interactions has not been formally explored (*24, 25, 31*). With approximately 3.6×10^6 heparin binding sites/cell, it might be expected that the level of salt-releasable binding of bFGF to heparin-treated cells would increase. This was not the case, however, as heparin was shown to inhibit salt-releasable bFGF binding in all cases, suggesting that the majority of the heparin bound to the cell surface was unable to bind bFGF. Since bFGF binding requires sulfate residues on heparin, it is likely that these sites were blocked on heparin when it was bound to cells. Thus, for the majority of the heparin binding sites on the cell surface, heparin−cell binding and heparin−bFGF binding might be mutually exclusive. Furthermore, heparin has been demonstrated to bind to components of the extracellular matrix, such as colla-
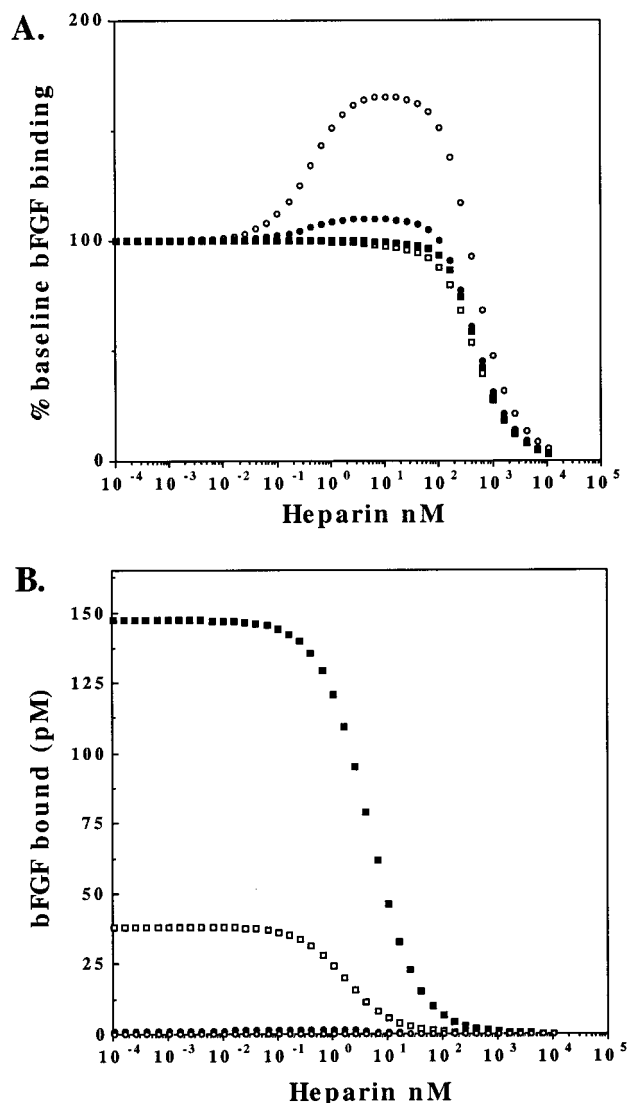
A.

B.

FIGURE 8: Model predictions of the effect of heparin on bFGF binding. Mathematical modeling based on previous work (*35*) was used to investigate how cellular binding of heparin, in the absence of cellular trafficking (4 °C), would impact bFGF binding to its receptor. Simulations were run at the indicated soluble heparin concentrations. (A) The theoretical data output from the simulations represent receptor-bound bFGF following exposure for 2.5 h to 0.22 nM bFGF and heparin (10^{-4}–10^5 nM) at normal levels of HSPG (100%) (■) and with increasing level of chlorate resulting in 10% (□), 1% (●), and 0.1% (○) of the normal levels of HSPG. (B) Data represent the amount of bFGF bound to cell-associated HSPG following exposure for 2.5 h to 0.22 nM bFGF and heparin (10^{-4}–10^5 nM) at normal levels of HSPG (100%) (■) and with increasing levels of chlorate resulting in 10% (□), 1% (●), and 0.1% (○) of the normal levels of HSPG.

gen type I and fibronectin (*31*), suggesting that a significant amount of the bound heparin might not be available to participate in cell surface events. Since no receptor was isolated in this study, it is not possible to identify the heparin binding site as a receptor. Given the high negative charge of heparin, it is likely that heparin is capable of forming a number of ionic interactions at the cell surface (*24, 25, 31*). What is recorded in the binding data of this study is the sum of all the interactions in which heparin takes part. The optimal heparin concentration required to enhance bFGF binding (approximately 5.6 nM) does not correlate directly

with the heparin binding curve (average $K_d = 54$ nM); however, it is possible that a subset of this heparin binding population is capable of interactions with bFGF and its receptors.

The increase in the level of bFGF–receptor binding in chlorate-treated cells in the presence of heparin was revealed only when a combination of high-salt and low-pH washes was used (Figure 4). This suggests that the increased level of binding resulted from the generation of heparin–bFGF–receptor complexes. The existence of a ternary complex involving bFGF, its receptor, and HSPG has been suggested previously by a number of groups both as a stabilizing arrangement for an increased level of bFGF binding and as an obligatory complex for bFGF activity (*4–7, 9, 13, 19, 43–46*). The study presented here suggests that these complexes formed with added heparin in cells whose HSPG sites were eliminated, resulting not only in an increased level of bFGF binding but also in increased levels of tyrosine phosphorylation and DNA synthesis.

The MAP kinase pathway has been shown to be active in bFGF signaling. In this study, with the bFGF concentrations that were used, MAP kinase stimulation was relatively insensitive to added heparin (Figure 7). A maximal response might be expected for control cells at the bFGF concentration that was used (0.22 nM), but it would be less likely for chlorate-treated cells since this dose was significantly below saturation at stimulating DNA synthesis (Figure 5). The MAP kinase pathway is one of a number of signaling processes that occur with bFGF stimulation (*1*). Thus, while activation of MAP kinase might have reached maximal levels, additional necessary pathways may not have been activated sufficiently to induce mitogenesis in chlorate-treated cells. However, it is important to note that we did not investigate the dynamics of MAP kinase activation in heparin-treated cells; thus, changes in the kinetics of MAP kinase activation might have occurred that are critical to the end point cellular response.

The addition of heparin to chlorate-treated cells was able to restore the bFGF-mediated stimulation of DNA synthesis to the levels observed with control cells (Figure 5). Since heparin has been shown to protect bFGF from proteolysis and physical denaturation, it is possible that increased bFGF activity in the presence of heparin could be explained by these effects. If this was the case, however, then heparin would also be expected to enhance bFGF activity in control cells as well. This was not observed. The fact that binding and signaling assays were conducted on time scales where significant bFGF degradation would not occur demonstrates that heparin enhanced bFGF binding and signaling independent of its physical stabilizing effects. In a previous study by Krufka et al. (*21*), high concentrations of heparin inhibited bFGF binding but not bFGF-mediated stimulation of DNA synthesis. These results suggested that a subclass of mitogenically active bFGF–FGFR complexes exist that are stabilized by heparin even under conditions where the majority of the bFGF–FGFR binding is inhibited by heparin. However, this previous study and the study presented here are complicated by the possibility that the dynamics of bFGF–heparin complex interactions both in solution and on the cell surface could result in a change in the kinetics for FGFR activation. Thus, heparin could initially inhibit bFGF binding yet enhance long-term FGFR activation by limiting

downregulation of receptor expression and signaling. These types of complex processes have been demonstrated to have a critical impact on the long-term biological response to epidermal growth factor and transforming growth factor α (47, 48). A detailed analysis of the kinetics of the various interactions of bFGF with its receptors and heparin in vitro has revealed that bFGF is potentially involved in forming a large number of different types of complexes at the cell surface (12). The study presented here suggests that additional heparin−cell surface interactions also need to be considered for this complex ligand−receptor system to be understood.

In this study, it was observed that bFGF binding, as well as bFGF-induced signaling and mitogenic response, was sensitive to HSPG and heparin within a range of physiologically relevant concentrations. These data suggest that conflicting results in the literature of stimulation and inhibition of bFGF by heparin might result from differences in doses and time courses used in the various studies. This could be important in designing parameters for the clinical use of heparin and heparin analogues as well as growth factor therapy. We evaluated the importance of heparin−cell surface binding through experiments and mathematical modeling. Using experimental conditions, computer simulations were able to replicate binding profiles similar to those obtained experimentally. Although the chemical nature and dynamics of these interactions clearly play important roles in dictating the ultimate biological response, the simple physical binding models suggest the significance of heparin−cell surface interactions in driving cellular response.

APPENDIX

Equations 1−12 describe the full heparin model system that was used to generate Figure 8.

$$\frac{dR}{dt} = -k_f^{CSR}LR + k_r^{CSR}C + k_{off}^T T - k_r^P GF_\infty(R,R^H)$$
$$\left(\frac{R}{R+R^H}\right) - k_{off}^T TF_\infty(R,P,R^H)\left(\frac{R}{R+P+R^H}\right) -$$
$$K_c^R G - k_f^{HR}\frac{H}{V}R + k_r^{HR1}R^H - k_r^{HR2}YF_\infty(R) - k_f^{SR}\frac{S}{V}R -$$
$$k_r^T T^H F_\infty(R,P)\left(\frac{R}{R+P}\right) \quad (1)$$

$$\frac{dG}{dt} = k_f^P LP - k_r^P G + k_r^{CSR}CF_\infty(P,R^H)\left(\frac{P}{P+R^H}\right) +$$
$$k_{off}^T TF_\infty(R,P,R^H)\left(\frac{P}{R+P+R^H}\right) - K_c^R G +$$
$$k_r^T T^H F_\infty(P,R)\left(\frac{P}{R+P}\right) \quad (2)$$

$$\frac{dT}{dt} = -k_{off}^T T + K_c^R G + K_c^P C \quad (3)$$

$$\frac{dT^H}{dt} = k_f^T LR^H - k_r^T T^H + k_r^{CSR}CF_\infty(P,R^H)\left(\frac{R^H}{P+R^H}\right) +$$
$$k_{off}^T TF_\infty(R,P,R^H)\left(\frac{R^H}{R+P+R^H}\right) +$$
$$k_r^P GF_\infty(R,R^H)\left(\frac{R^H}{R+R^H}\right) + k_f^{SR}\frac{S}{V}R \quad (4)$$

$$\frac{dR^H}{dt} = -k_f^T LR^H - k_r^T T^H - k_r^{CSR}CF_\infty(P,R^H)\left(\frac{R^H}{P+R^H}\right) -$$
$$k_{off}^T TF_\infty(R,P,R^H)\left(\frac{R^H}{R+P+R^H}\right) -$$
$$k_r^P GF_\infty(R,R^H)\left(\frac{R^H}{R+R^H}\right) + k_f^{HR}\frac{H}{V}R - k_r^{HR1}R^H +$$
$$k_r^{HR2}YF_\infty(R) \quad (5)$$

$$\frac{dH}{dt} = -k_{on}^H HL + k_{off}^H S - k_f^{HR}\frac{H}{V}R + k_r^{HR1}R^H[1 - F_\infty(Q)] -$$
$$k_f^{HR}\frac{H}{V}Q + k_r^{HR2}Y[1 - F_\infty(R)] \quad (6)$$

$$\frac{dY}{dt} = k_f^{HR}\frac{H}{V}Q - k_r^{HR2}Y + k_r^{HR1}R^H F_\infty(Q) \quad (7)$$

$$vL = vL_o - T - C - G - T^H - S \quad (8)$$

$$P = P_o - T - G \quad (9)$$

$$C = R_o = T - R - R^H - T^H \quad (10)$$

$$S = H_o - H - R^H - T^H - Y \quad (11)$$

$$Y = Q_o - Q \quad (12)$$

where L is [bFGF], R is [FGFR], C is [L−R], P is [HSPG], G is [L−P], T is [L−P−R], H is [heparin], S is [L−H], Q is [heparin receptors], R^H is [H−R], Y is [H−Q], T^H is [L−H−R], and $F_\infty(X)$ is the probability of capture of the dissociated ligand by X (35). k_{off}^X and k_{on}^X are the intrinsic association and dissociation rate constants, respectively. K_c^R and K_c^P are the surface diffusion-limited coupling rate constants. k_f^X and k_r^X are the apparent forward and reverse binding rate constants, respectively:

$$k_f^{CSR} = \frac{k_{on}^{CSR}}{1 + \frac{k_{on}^{CSR}R + k_{on}^P P}{4\pi aD}}; k_f^P = \frac{k_{on}^P}{1 + \frac{k_{on}^{CSR}R + k_{on}^P P}{4\pi aD}};$$

$$k_f^{SR} = \frac{k_{on}^*}{1 + \frac{k_{on}^* R}{4\pi aD}}; k_f^{HR} = \frac{k_{on}^{HR}}{1 + \frac{k_{on}^{HR}R + k_{on}^{HR}Q}{4\pi aD_H}};$$

$$k_r^{CSR} = \frac{k_{off}^{CSR}}{1 + \frac{k_{on}^{CSR}R}{4\pi aD + k_{on}^P P}};$$

$$k_r^P = \frac{k_{off}^P}{1 + \frac{k_{on}^P P}{4\pi aD + k_{on}^{CSR}R}}; k_r^{SR} = \frac{k_{off}^*}{1 + \frac{k_{on}^* R}{4\pi aD}};$$

$$k_r^{HR1} = \frac{k_{off}^{HR}}{1 + \frac{k_{on}^{HR}R}{4\pi aD_H + k_{on}^{HR}Q}}; k_r^{HR2} = \frac{k_{off}^{HR}}{1 + \frac{k_{on}^{HR}Q}{4\pi aD_H + k_{on}^{HR}R}}$$

The addition of heparin results in four differential equations (eqs 4−7) and two algebraic constraints (eqs 11 and 12) related to the concentrations of unbound heparin (H), bFGF−

Table 3

parameter	value		ref
k_{on}^{CSR}	2.5×10^8 M^{-1} min^{-1}		7
k_{off}^{CSR}	0.048 min^{-1}		7
k_{on}^*	2.27×10^8 M^{-1} min^{-1}		7
k_{off}^*	0.003 min^{-1}		7
k_{on}^P	0.9×10^8 M^{-1} min^{-1}		7
k_{off}^P	0.068 min^{-1}		7
k_{on}^{HR}	$=k_{on}^P$		
k_{off}^{HR}	$=k_D^{HR}k_{on}^{HR}$ where $k_D^{HR} = 54$ nM	experimentally determined	
k_{on}^H	$=k_{on}^P$		
k_{off}^H	$=k_D^H k_{on}^H$ where $k_D^H = 1$ nM		25
R_o, Q_o, P_o	1.5×10^4 receptors/cell		9
	1.5×10^6 sites/cell	experimentally determined	
	$3.6 \times 10^{-6} - R_o$ sites/cell		
K_c^R	function(G,R,k_{off}^P,D_G)		35
K_c^P	function(C,P,k_{off}^{CSR},D_C)		35
D, D_H	1×10^{-6} cm^2 s^{-1}	diffusion coefficient for bFGF, heparin in solution (estimate based on size)	
D_G, D_C	1×10^{-9} cm^2 s^{-1}	diffusion coefficient for CSR, HSPG in the membrane (estimate based on size)	
a	5×10^{-6} m	cell radius	
V	1×10^{-6} mL/cell	cell volume	

heparin solution complex (S), unbound (Q) and bFGF-bound (Y) heparin surface receptors, heparin–FGFR complexes (R^H), and bFGF–heparin–FGFR triads (T^H) as well as appropriate changes reflecting the inclusion of heparin in the base model equations (eqs 1–3 and 8–10). Chlorate treatment resulting in the loss of salt-sensitive binding was manifested in the model as the elimination of eqs 2, 3, and 9 and the corresponding terms in the remaining equations. Reduced chlorate concentrations were reflected in altered initial conditions with respect to the level of HSPG binding sites (P_o).

The parameters included in the mathematical model were determined experimentally in the current study (i.e., heparin binding affinity for cell surface binding sites) or were determined in previous studies. Some parameters represent theoretical estimates. The parameters that were used are listed in Table 3.

ACKNOWLEDGMENT

REFERENCES

1. Bikfalvi, A., Klein, S., Pintucci, G., and Rifkin, D. B. (1997) *Endocr. Rev. 18*, 26–45.
2. Conrad, E. H. (1998) *Heparin-binding proteins*, Academic Press, San Diego.
3. Schlessinger, J., Lax, I., and Lemmon, M. (1995) *Cell 83*, 357–360.
4. Turnbull, J. E., and Gallagher, J. T. (1993) *Biochem. Soc. Trans. 21*, 477–482.
5. Yayon, A., Klagsbrun, M., Esko, J. D., Leder, P., and Ornitz, D. M. (1991) *Cell 64*, 841–848.
6. Rapraeger, A., Krufka, A., and Olwin, B. (1991) *Science 252*, 1705–1708.
7. Nugent, M. A., and Edelman, E. R. (1992) *Biochemistry 31*, 8876–8883.
8. Roghani, M., Mansukhani, A., Dell'Era, P., Bellosta, P., Basilico, C., Rifkin, D. B., and Moscatelli, D. (1994) *J. Biol. Chem. 269*, 3976–3984.
9. Fannon, M., and Nugent, M. A. (1996) *J. Biol. Chem. 271*, 17949–17956.
10. Olwin, B. B., and Rapraeger, A. (1992) *J. Cell Biol. 118*, 631–639.
11. Kan, M., Wang, F., Xu, J., Crabb, J. W., Hou, J., and McKeehan, W. L. (1993) *Science 259*, 1918–1921.
12. Pantoliano, M. W., Horlick, R. A., Springer, B. A., Van Dyk, D. E., Tobery, T., Wetmore, D. R., Lear, J. D., Nahapetian, A. T., Bradley, J. D., and Sisk, W. P. (1994) *Biochemistry 33*, 10229–10248.
13. Forsten, K. E., Courant, N. A., and Nugent, M. A. (1997) *J. Cell. Physiol. 172*, 209–220.
14. Nugent, M. A., Karnovsky, M. J., and Edelman, E. R. (1993) *Circ. Res. 73*, 1051–1060.
15. Turnbull, J. E., Fernig, D. G., Ke, Y., Wilkinson, M. C., and Gallagher, J. T. (1992) *J. Biol. Chem. 267*, 10337–10341.
16. Habuchi, H., Suzuki, S., Saito, T., Tamura, T., Harada, T., Yoshida, K., and Kimata, K. (1992) *Biochem. J. 285*, 805–813.
17. Walker, A., Turnbull, J. E., and Gallagher, J. T. (1994) *J. Biol. Chem. 269*, 931–935.
18. Maccarana, M., Casu, B., and Lindahl, U. (1993) *J. Biol. Chem. 268*, 23898–23905.
19. Guimond, S., Maccarana, M., Olwin, B. B., Lindahl, U., and Rapraeger, A. C. (1993) *J. Biol. Chem. 268*, 23906–23914.
20. Tyrrell, D. J., Ishihara, M., Rao, N., Horne, A., Kiefer, M. C., Stauber, G. B., Lam, L. H., and Stack, R. J. (1993) *J. Biol. Chem. 268*, 4684–4689.
21. Krufka, A., Guimond, S., and Rapraeger, A. (1996) *Biochemistry 35*, 11131–11141.
22. Mali, M., Elenius, K., Miettinen, H. M., and Jalkanen, M. (1993) *J. Biol. Chem. 268*, 24215–24222.
23. Hondermarck, H., Deudon, E., and Boilly, B. (1992) *Dev. Brain Res. 68*, 247–253.
24. Letourneur, D., Caleb, B. L., and Castellot, J. J. (1995) *J. Cell. Physiol. 165*, 676–686.
25. Castellot, J. J., Wong, K., Herman, B., Hoover, R. L., Albertini, D. F., Wright, T. C., Caleb, B. L., and Karnovsky, M. J. (1985) *J. Cell. Physiol. 124*, 13–20.
26. Glimelius, B., Busch, C., and Hook, M. (1978) *Thromb. Res. 12*, 773–782.
27. Patton, W. A., Granzow, C. A., Getts, L. A., Thomas, S. C., Zotter, L. M., Gunzel, K. A., and Lowe-Krentz, L. J. (1995) *Biochem. J. 311*, 461–469.
28. Sasisekharan, R., Bulmer, M., Moremen, K., Cooney, C., and Langer, R. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 3660–3664.
29. Nugent, M. A., and Edelman, E. R. (1992) *J. Biol. Chem. 267*, 21256–21264.
30. Connolly, D. T., Knight, M. B., Harakas, N. K., Wittwer, A. J., and Feder, J. (1986) *Anal. Biochem. 152*, 136–140.
31. San Antonio, J. D., Slover, J., Lawler, J., Karnovsky, M. J., and Lander, A. D. (1993) *Biochemistry 32*, 4746–4755.
32. Bassols, A., and Massagué, J. (1988) *J. Biol. Chem. 263*, 3039–3045.
33. Moscatelli, D. (1987) *J. Cell. Physiol. 131*, 123–130.
34. Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660–672.
35. Forsten, K. E., and Lauffenburger, D. A. (1994) *Mol. Immunol. 31*, 739–751.
36. Hindmarsh, A. (1980) *ACM-Signum Newsletter 15*, 10–11.
37. Press, W. H., Flannery, B. P., Teukolsky, S. A., and Vetterling, W. T. (1986) *Numerical Recipes*, Cambridge University Press, Cambridge, U.K.
38. Ornitz, D. M., Yayon, A., Flanagan, J. G., Svahn, C. M., Levi, E., and Leder, P. (1992) *Mol. Cell. Biol. 12*, 240–247.
39. Ornitz, D. M., Herr, A. B., Nilsson, M., Westman, J., Svahn, C.-M., and Waksman, G. (1995) *Science 268*, 432–436.

40. Kjellen, J., Pettersson, I., and Hook, M. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 5371−5375.

41. Lapierre, F., Holme, K., Lam, L., Tressler, R. J., Storm, N., Wee, J., Stack, R. J., Castellot, J., and Tyrrell, D. J. (1996) *Glycobiology 6*, 355−366.

42. Bernfield, M., and Hooper, K. C. (1991) *Ann. N.Y. Acad. Sci. 638*, 182−194.

43. Gospodarowicz, D., and Cheng, J. (1986) *J. Cell. Physiol. 128*, 475−484.

44. Klagsbrun, M., and Baird, A. (1991) *Cell 67*, 229−231.

45. Moscatelli, D., and Devesly, P. (1990) *Growth Factors 3*, 25−33.

46. Ornitz, D. M., and Leder, P. (1992) *J. Biol. Chem. 267*, 16305−16311.

47. Reddy, C. C., Niyogi, S. K., Wells, A., Wiley, H. S., and Lauffenburger, D. A. (1996) *Nat. Biotechnol. 14*, 1696−1699.

48. Reddy, C. C., Wells, A., and Lauffenburger, D. A. (1996) *J. Cell. Physiol. 166*, 512−522.