Diminished Heparin Binding of a Basic Fibroblast Growth Factor Mutant Is Associated with Reduced Receptor Binding, Mitogenesis, Plasminogen Activator Induction, and *in Vitro* Angiogenesis†

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ABSTRACT: Using modeling of heparin–fibroblast growth factor interactions, we replaced four basic residues of basic fibroblast growth factor (FGF-2) with neutral glutamine residues by site-specific mutagenesis to give the mutants K128Q, K138Q, K128Q-K138Q, R129Q, K134Q, and R129Q-K134Q. The FGF mutants were characterized for their receptor and heparin binding affinities, mitogenic and cell proliferation activities, and their ability to induce plasminogen activator (PA) production and *in vitro* angiogenesis by cultured endothelial cells. Heparin binding properties and biological activities of the three mutants involving R129 and K134 remained essentially unchanged; however, significant changes for three mutants involving K128 and K138 were found. The K_D values for heparin binding for K128Q and K138Q mutants were increased about 10-fold, and that for the K128Q-K138Q double mutant was increased by about 100-fold. The mutant K128Q-K138Q required a 10-fold higher concentration of heparin to promote binding to heparan sulfate proteoglycan (HSPG)-deficient CHO cells transfected with fibroblast growth factor receptor-1 (FGFR1) or to induce DNA synthesis in HSPG-deficient myeloid cells transfected with FGFR1. Binding affinities of the mutants to cell surface receptors on BHK-21 cells, however, were similar to that of wild-type FGF-2. In endothelial cell proliferation assays the activities of K128Q and K128Q-K138Q were about 10-fold lower than that of the wild-type protein, whereas the K138Q mutant exhibited wild-type activity. In addition, the K128Q-K138Q mutant displayed a markedly lowered capacity to induce PA activity in cultured endothelial cells and to form capillary-like structures in an *in vitro* angiogenesis model. These findings are in accord with the view that residues K128 and K138 are critical components of the heparin binding site of FGF-2 and that diminished heparin binding is associated with a reduction in the biological properties of FGF.

Modulation of growth factor activity by cell-associated proteoglycans is evolving as a central theme in the regulation of cell growth (Nathan & Sporn, 1991; Ruoslahti & Yamaguchi, 1991). Basic fibroblast growth factor (FGF-2),[1] one of a nine-member family of polypeptides (Miyamoto et al., 1993), exhibits angiogenic and a variety of growth and differentiation activities (Baird & Böhlen, 1990; Basilico & Moscatelli,1992). FGF's are characterized by their high affinity toward heparan sulfate proteoglycans (HSPG). HSPGs are a highly diverse group of glycoproteins composed of a core protein to which the sulfated glycosaminoglycan, heparan sulfate, is covalently attached (Yanagishita & Hascall, 1992). Various roles for the interaction of HSPGs with the prototypic members, FGF-1 and FGF-2, have been proposed, including physical stabilization of the protein (Gospodarowitz & Chang, 1986; Volkin et al., 1993a), protection from proteolytic degradation (Sommer & Rifkin, 1989), and extracellular storage of FGF (Vlodavsky et al., 1987). More recent studies have shown this interaction to be essential for the binding of FGF to its cell surface tyrosine kinase receptor (Yayon et al., 1991) and important in mediating internalization (Roghani & Moscatelli, 1992) and intracellular targeting (Reiland & Rapraeger, 1993). Although the saccharide and sulfation patterns in heparan sulfate are variable, heparan sulfates with specific activating and inhibitory sequences that bind FGF have been isolated (Turnbull et al., 1992; Habuchi et al., 1992; Ishihara et al., 1993; Ishai-Michaeli et al., 1992; Maccarana, et al., 1993; Guimond et al., 1993; Mali et al., 1993; Walker et al., 1994) and structural requirements for binding FGF-2 (Maccarana et al., 1993) and for receptor binding (Walker et al., 1994; Aviezer et al., 1994) have been deduced. Further, evidence that the response of neural cells to either FGF-1 or FGF-2 may be modulated during development by expression of differentially glycosylated forms of HSPG has been presented (Nurcombe et al., 1993).

Many of the biological properties of proteoglycans are ascribed to interactions between highly negatively charged glycosaminoglycan chains and positively charged side chains of proteins (Kjellén et al., 1991). The nature of these interactions can range from nonspecific electrostatic association to those that are highly specific, such as the interaction of heparin with antithrombin (Lindahl, 1986). The binding sites for heparin/heparan sulfate on FGF have not been defined. Although heparin-binding peptide fragments of FGF-2 have been identified (Baird et al., 1988) and limited structure–function studies on the protein have been conducted (Seddon et al., 1991; Seno et al., 1990; Heath et al., 1991), we found that an intact three-dimensional structure is required for high-affinity binding of FGF-2 to heparin (Seddon et al.,

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[1] Abbreviations: FGF-2, fibroblast growth factor-2; FGF, fibroblast growth factor; FGFR1, fibroblast growth factor receptor-1; HSPG, heparan sulfate proteoglycans; BCDS, β-cyclodextrin tetradecasulfate; BHK cells, baby hamster kidney cells; ABAE cells, bovine aortic arch endothelial cells; CHO cells, Chinese hamster ovary cells; DMEM, Dulbecco's modified Eagle's medium; PA, plasminogen activator; EC_{50}, half-maximal stimulatory concentration; SOS, sucrose octasulfate.

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1991). From the crystal structure of FGF-2 (Eriksson et al., 1991; Zhang et al., 1991), two pairs of basic residues, K128 and K138, and R129 and K134, respectively, were found to form binding sites for two sulfate molecules. It is plausible that these residues are involved in the binding of the sulfate moieties of heparin and cell surface HSPGs implicated in modulating the biological activities of FGF-2. To assess the role of these basic residues in FGF–heparin interactions and the impact of the neutralization of positively charged side chains on the biological activities of FGF-2, site 1 (K128 and K138) and site 2 (R129 and K134) were replaced with glutamine residues by site-directed mutagenesis to give mutants K128Q, K138Q, K128Q-K138Q, R129Q, K134Q, and R129Q-K134Q. The mutants were examined for their abilities to interact with heparin and cell surface receptors using several experimental approaches, for their abilities to induce proliferation and plasminogen activator production in cultured adult bovine aortic endothelial (ABAE) cells, and for formation of capillary-like structures by ABAE cells cultured on three-dimensional collagen gels. Our studies demonstrate that neutralization of charge at residues K128 and K138 (site 1) is associated with a significant reduction in the affinity of the protein for heparin and biological activities of FGF.

EXPERIMENTAL PROCEDURES

Construction of Human FGF-2 Mutants. The gene encoding human E^3, E^5 FGF-2 (Seddon et al., 1991) was cloned into the T7 expression vector pET(M13)ΔPS, a derivative of pET-3a (Rosenberg et al., 1987), between restriction sites *Nde*1 and *Bam*H1. The plasmid was modified by site-directed mutagenesis using single-stranded DNA and two primers, 5′-GTCTAGAAAA TACACCAGTT GGTACGTAGC ACTGCAGCGA ACCGGTCAGT ATAAA-3′ and 5′-CTTGGTTCCC AAACAGGGCC CGGGCAGAAA GCTA-3′, to obtain the K128Q mutant gene, which contains the newly introduced unique restriction sites *Sna*B1, *Pst*1, and *Apa*1 (underlined), respectively. Cassette-directed mutagenesis of this variant gene using *Sna*B1 and *Apa*1, and two sets of annealed oligonucleotides, 5′-GTAGCACTTA AGCGTACGGG GCAGTATAAG CTTGGTTCCC AAACAGGGCC-3′ and 5′-CTGTTTGGGA ACCAAGCTTA TACTGCCCCG TACGCTTAAG TGCTAC-3′, and 5′-GTAGCACTGC AGCGTACGGG GCAGTATAAG CTTGGTTCCC AAACAGGGCC-3′ and 5′-CTGTTTGGGA ACCAAGCTTA TACTGCCCCG TACGCTGCAG TGCTAC-3′, gave, respectively, a mutant K138Q which has new restriction sites *Afl*2 and *Hin*d3 (underlined) and a double mutant K128Q-K138Q which has new restriction site *Hin*d3 (underlined). Cassette mutagenesis of the K128Q-K138Q mutant using unique restriction sites *Sna*B1 and *Apa*1 and annealed oligonucleotides 5′-GTAGCACTTA AGCAGACGGG GCAGTATCAG CTTGGTTCCA AAACAGGGCC-3′ and 5′-CTGTTTTGGA ACCAAGCTGA TACTGCCCCG TCTGCTTAAG TGCTAC-3′ produced another double mutant R129Q-K134Q, which has a new restriction site *Afl*2 (underlined). This mutant was further used to generate two more variants, again by cassette-directed mutagenesis, using sites *Afl*2 and *Apa*1 and annealed oligonucleotides 5′-TTAAGCAGAC GGGGCAGTAT AAGCTTGGTT CCAAAACAGG GCC-3′ and 5′-CTGTTTTGGA ACCAAGCTTA TACTGCCCCG TCTGC-3′, to give mutant R129Q with a new restriction site *Hin*d3 (underlined), and 5′-TTAAGCGTAC GGGGCAGTAT CAGCTTGGTT CCAAAACAGG GCC-3′ and 5′-CTGTTTTGGA ACCAAGCTGAT ACTGCCCCGT ACGC-3′, to give K134Q with new restriction site *Spl*1 (underlined). The desired mutant plasmids were selected for on the basis of susceptibility to cleavage at the newly introduced restriction sites and confirmed by complete sequencing of the genes.

Expression and Purification of the Mutants. The plasmids containing the genes encoding the seven mutants as described above were transformed into competent *Escherichia coli* BL21 plys S and cultured at 37 °C in Luria broth containing 50 μg/mL ampicillin and 30 μg/mL chloramphenicol until an absorbance at 600 nm of 0.4 was reached. Expression of the recombinant protein was induced by the addition of 2 mM isopropyl thiogalactoside for 2 h at 37 °C. Cells from a 1-L culture were harvested by centrifugation, resuspended in 30 mL of 50 mM Tris-HCl, pH 7.5, containing 0.1 mM EDTA and 0.6 M NaCl, and disrupted by treatment with lysozyme (10 μg/mL) for 20 min at 4 °C followed by sonication (6 × 30 s pulses). The lysates were clarified by centrifugation (10000*g*; 20 min) and the supernatant solutions incubated with 5 mL of hydrated heparin–Sepharose (Pharmacia/LKB) at 4 °C for 1 h with constant rotation. The resin was isolated by filtration on 0.8-μm filter apparatus (Nalgene) and washed extensively with 10 mM Tris-HCl, pH 7.4, containing 0.6 M NaCl, and bound protein was eluted with Tris buffer containing 3 M NaCl (25 mL). The 3 M NaCl eluent was diluted 6-fold with Tris buffer and loaded onto a TSK Heparin-5PW column (0.75 × 7.5 cm; The Nest Group, MA) equilibrated with 10 mM Tris and 0.6 M NaCl (pH 7.4). Elution of bound material was monitored at 280 nm and was accomplished using a linear salt gradient (0.6–3.0 M NaCl in 60 min) at a flow rate of 0.7 mL/min.

Determination of Thermodynamic Dissociation Constants (K_D). Fluorescence titration measurements were conducted in a Perkin-Elmer LS-5 fluorospectrometer using excitation and emission wavelengths of 290 and 350 nm, respectively. Two milliliters of FGF solution in 10 mM Tris-HCl, pH 7.4, containing 2 mM dithiothreitol, was placed in a cuvette. An aliquot (2–5 μL) of heparin (Hepar Industries, Franklin, OH), β-cyclodextrin tetradecasulfate (BCDS), or (2-hydroxypropyl)-β-cyclodextrin (gifts from George Reed, American Maize-Products, Hammond, IN) was added to the cuvette and the fluorescence intensity of the solution read against a solution of tryptophan matched to gave comparable fluorescence intensity and to which an identical amount of heparin or BCDS was added. Fluorescence titration measurements were repeated at least twice. Briefly, analysis of the fluorescence titration data (L.-Y. Li and A. P. Seddon, unpublished data) assumes that one heparin fragment binds to one FGF molecule, and the change in the fluorescence emission is described by the equation (Taira & Benkovic, 1987):

$$F_O = F_I + \Delta F[([P]_t + K_D + [L]_t) - \{([P]_t + K_D + [L]_t)^2 - 4[P]_t[L]_t\}^{1/2}]/(2[P]_t) \quad (1)$$

where F_O is the observed fluorescence intensity, F_I is the initial fluorescence intensity, ΔF is the final change in fluorescence intensity, K_D is the equilibrium dissociation constant, and $[P]_t$ and $[L]_t$ are the total concentration of the protein and the ligand, respectively. When the total protein concentration $[P]_t$ is much greater than K_D, eq 1 can be simplified to give the equation:

$$F_O = F_I + \Delta F[L]_t/[P]_t \quad (2)$$

For each titration eq 2 was used to determine the FGF concentration, which is equal to the total concentration of the ligand ($[P]_t = [L]_t$) as the titration approaches saturation

($\Delta F = F_O - F_I$). For the case when $[P]_t$ is not much greater than K_D, the FGF concentration determined by amino acid analysis was used. The use of eq 1 assumes that the stoichiometry between FGF and heparin be 1:1. It is likely that more than one molecule of FGF may bind to a single heparin chain (Mach et al., 1993). Assuming that the affinities of the binding sites on the ligand toward FGF are similar, the presence of n binding sites on the ligand can be interpreted as the effective concentration of the ligand that is greater than the actual concentration of the ligand by a factor of n-fold. The use of eq 1 is still valid in this case if the factor n is incorporated into the equation so that $[L]_t$ now becomes $n[L]_t$, and the values of dissociation constants would be larger than those presented in Table 1 by n-fold. Caution should be exercised in drawing any conclusions on the stoichiometry of the FGF–heparin complex because of the heterogeneous nature of heparin, the presence of high- and low-affinity binding sites (Mach et al., 1993), and the possibility of induced aggregation of the protein when bound to heparin.

Receptor Binding. The mutant proteins were tested for their capacity to compete for the binding of ^{125}I-FGF-2 (Amersham Corp.) to BHK (baby hamster kidney) cells, which express high numbers of the FGF receptor, essentially as described (Moscatelli, 1987). Briefly, cells plated on 24-well plates were incubated with 50 pM ^{125}I-FGF-2 with serial dilutions of unlabeled FGF-2 or the mutants at room temperature for 1 h. The cells were then incubated at 4 °C for 30 min, washed twice with phosphate-buffered saline and treated with 20 mM Hepes, pH 7.5, containing 2 M NaCl to remove ^{125}I-FGF-2 bound to low-affinity HSPG binding sites. Receptor-bound FGF-2 was recovered by treatment of the cells with 0.5% Triton X-100 in 0.1 M sodium phosphate, pH 8, and counted in a γ-counter. Assays were conducted in duplicate. Receptor binding as a function of heparin concentration was examined using a CHO mutant cell line Pgs A745 (a gift from Dr. J. D. Esko, Department of Biochemistry, University of Alabama, Birmingham) deficient in xylosyltransferase (Esko, 1991) transfected with FGFR-1 as described (Yayon et al., 1991). The transfected cells were cultured in F12 medium supplemented with 10% fetal calf serum and exhibited high-affinity binding of FGF-2 in the presence of 40–200 ng/mL heparin. Receptor binding was performed as described (Yayon et al., 1991). Briefly, ^{125}I-labeled FGF-2 bound to HSPGs was released from the cell surface by a 5-min incubation with 20 mM HEPES buffer, pH 7.4, containing 1.6 M NaCl at 4 °C. Receptor-bound FGF-2 was determined by extraction with 20 mM acetate buffer, pH 4.0, containing 2 M NaCl. Radioactivity was quantitated using a γ-counter.

Endothelial Cell Proliferation and [^{3}H]Thymidine Incorporation Assays. The mitogenic activities of FGF-2 and mutants were determined using bovine vascular endothelial cells derived from adult aortic arch as described (Gospodarowicz et al., 1984). Briefly, cells were seeded at an initial density of 8000 cells per 24-well plate in 0.5 mL Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum (Hyclone, Logan, UT) supplemented with penicillin (100 units/mL), streptomycin (100 μg/mL), and L-glutamine (2 mM). Two hours after plating, 20-μL aliquots of serial dilutions of FGF-2 and the mutants in DMEM were added. After 5 days in culture, duplicate plates were trypsinized and cell densities determined by cell counting in a Coulter counter. Determinations were conducted in duplicate. Heparin-dependent FGF-induced incorporation of [^{3}H]thymidine into 32D myeloid cells transfected with FGFR-1 was determined essentially as described (Guimond et al., 1993). 32D-FGFR1 myeloid cells were maintained in Iscove's medium containing 10% fetal calf serum, penicillin (50 units/mL), and streptomycin (50 μg/mL) and supplemented with interleukin-3 every other day. Cells were serum starved for 24 h. FGF-2 was then added at 20 ng/mL, in the presence of heparin (0–500 μg/mL), and the cells were incubated for an additional 18 h. Following addition of [^{3}H]thymidine, incorporation into DNA was measured after a 6-h period and processed as described (Guimond et al., 1993).

Plasminogen Activator Induction Assay. ABAE cells were seeded at 20 000 cells/well in 96-well plates and maintained in DMEM containing 10% calf serum (Hyclone, Logan, UT) supplemented with penicillin (100 units/mL), streptomycin (100 μg/mL), and L-glutamine (2 mM) and different concentrations of FGF-2 or mutants. After 24 h, the cells were washed with PBS and lysed in 60 mM Tris-HCl, pH 8.5, containing 0.05% Triton X-100. Cell-associated plasminogen activator (PA) activity was measured as described (Presta et al., 1989) using the plasmin chromogenic substrate, D-norleucyl-hexahydrotyrosyl-lysine *p*-nitroanilide acetate, and read from a standard curve constructed using low molecular weight urokinase plasminogen activator (American Diagnostica, Greenwich, CT). One international unit (IU) of urokinase plasminogen activator activity, as supplied by the manufacturer, is referenced to the World Health Organization International Reference Plasma and was used to express PA activity. Cell-associated protein concentrations were determined using the method of Bradford (1976).

In Vitro Angiogenesis. Three-dimensional collagen gel plates (24-well) were prepared by addition of 0.5 mL of a chilled solution of 0.7 mg/mL of rat tail type I collagen (Becton Dickinson Labwares, Bedford, MA) containing 1× DMEM and adjusted to neutral pH with $NaHCO_3$ to each well. After formation of collagen gel (about 1–2-mm thickness), ABAE cells were seeded at 50 000 cells/well. The cultures were maintained at 37 °C in DMEM containing 10% calf serum (Hyclone, Logan, UT) supplemented with penicillin (100 units/mL), streptomycin (100 μg/mL), and L-glutamine (2 mM). Confluent cultures were achieved in about 5 days, by which time the cells formed a monolayer on the gel. The medium was then replaced with fresh medium containing different concentrations of FGF-2 or FGF-2 mutants. The cultures were maintained at 37 °C for 48 h and then discontinued by fixation with cold methanol (–20 °C). The gels were then soaked in PBS/glycerol (1:1) solution and transferred onto glass slides. The extent of capillary-like structure formation by ABAE cells was examined by using the technique of computer-assisted image analysis, using a Kontron IBAS Image Analyser assisted with a Hamamatsu C2400 video camera and a Zeiss Axioshop microscope. The video image of the capillary-like tubes formed by ABAE cells that invaded in the collagen gel is converted to white areas, whereas the monolayer of quiescent ABAE cells on the surface of the gel is converted into a black background. The ratio of the white areas over the total area observed is calculated to quantitate the extent of the tube formation. Three culture wells were used for each sample, and three microscopic fields were examined for each well. Each experimental point represents results from examination of nine microscopic fields.

RESULTS

A series of FGF's with point mutations in the putative heparin binding site on FGF-2 were expressed in *E. coli* and purified to apparent homogeneity from the soluble fraction of *E. coli* extracts by heparin affinity chromatography as

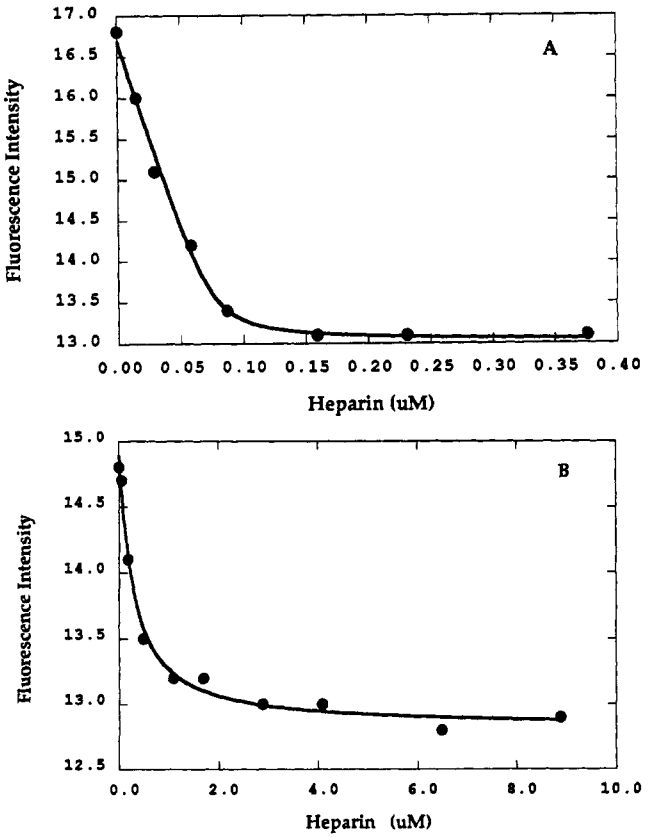

FIGURE 1: Effect of heparin on the intrinsic fluorescence of FGF-2 and K128Q-K138Q mutant. Fluorescence intensities for FGF-2 (panel A) and K128Q-K138Q (panel B) as a function of heparin concentration were recorded as described under Experimental Procedures. The buffer was 10 mM Tris-HCl, pH 7.4, containing 2 mM dithiothreitol, and the concentrations of FGF-2 and K128Q-K138Q were 370 and 280 nM, respectively.

previously described (Seddon et al., 1991). Introduction of mutations was confirmed by complete sequencing of the gene and analysis of the proteins was by SDS–PAGE, reverse-phase HPLC, and N-terminal sequence and amino acid analysis. Attempts were made to isolate a quadruple mutant, K128Q-K134Q-K138Q-R129Q, which was expressed at levels comparable to that of the other mutants but was confined to the inclusion bodies. Solubilization, refolding, and purification the protein, using a variety of extraction and purification procedures, failed to yield sufficient material for analysis and was not pursued further. The impact of the substitutions of basic amino acid side chains for neutral glutamine residues on heparin and receptor binding, and on the biological properties of FGF-2, was assessed.

Quench of Intrinsic Fluorescence of FGF. FGF-2 contains a single tryptophan residue at position 123. Crystallographic data suggest that W123 is located close to a cluster of basic residues implicated to be involved in heparin binding (Eriksson et al., 1991; Zhang et al., 1991; Zhu et al., 1991), suggesting that the fluorescence emission of W123 may be used to report local changes in protein conformation upon binding various ligands. We, therefore, evaluated the fluorescence properties of FGF-2 in the presence and absence of heparin and a heparin mimic, β-cyclodextrin tetradecasulfate (BCDS). The addition of heparin to a solution of FGF-2 resulted in a concentration-dependent quench of the tryptophan fluorescence emission (Figure 1A). Addition of BCDS to FGF-2 also resulted in a quenching of the fluorescence (data not shown), whereas addition of a nonsulfated analog, (2-hydroxpropyl)-β-cyclodextrin, showed no spectral effects (data not shown).

Table 1: Thermodynamic Dissociation Constants (K_D) and Corresponding Free Energy Changes ($\Delta G°$) Associated with the Binding of Heparin and BCDS to FGF-2 and Mutant Proteins

	heparin		BCDS	
FGF	K_D (nM)	$\Delta G°$ (kcal/mol)	K_D (nM)	$\Delta G°$ (kcal/mol)
FGF-2	2.3 ± 0.9	−11.78	9.2 ± 1.7	−10.96
K128Q	19.3 ± 1.8	−10.52	29.1 ± 8.2	−10.27
K138Q	10.8 ± 6.9	−10.86	22.2 ± 12.4	−10.44
K128Q-K138Q	247.7 ± 116.4	−9.01	534.5 ± 93.5	−8.55
R129Q	0.5 ± 0.4	−12.68	0.5 ± 0.2	−12.68
K134Q	0.4 ± 0.1	−12.81	3.5 ± 0.1	−11.53
R129Q-K134Q	1.7 ± 1.1	−11.96	30.7 ± 2.1	−10.24

Determination of Thermodynamic Dissociation Constants for Heparin Binding. Thermodynamic dissociation constants (K_D) obtained from fluorescence quenching experiments for wild-type FGF-2 and the mutant proteins using heparin and BCDS and the corresponding free energy changes ($\Delta G°$) are shown in Table 1. The K_D value for the binding of heparin to the wild-type FGF-2 is in good agreement with published estimates of the dissociation constants [2 nM, Moscatelli (1987); and 7.5 nM, Nugent and Edelman (1992)] obtained by analysis of FGF-2 bound to cell-associated or isolated heparan sulfate proteoglycans. As revealed by fluorescence titration studies, the two single-residue mutants, K128Q and K138Q, exhibited about a 10-fold decrease in affinity toward heparin, corresponding to a destablization change of free energy change ($\Delta\Delta G°$, calculated according to the relationship: $\Delta\Delta G° = -RT \ln[K_{D(mutant)}/K_{D(wt)}]$) of +1.4 kcal/mol, whereas the double mutant, K128Q-K138Q, showed a marked 100-fold decrease in heparin binding, corresponding to a change of free energy change of +2.7 kcal/mol. That the change of free energy change associated with the diminished affinity of the mutants toward heparin is additive indicates that 128 and 138 interact with heparin in an independent manner. Conversely, substitutions at R129 or K134 with glutamine lead to a slight decrease in the K_D for both heparin and BCDS. Interestingly, the double mutant R129Q-K134Q gave a value similar to that of FGF-2. Of note is that during purification of the proteins the concentration of NaCl required to elute FGF-2 and the mutant proteins from heparin–Sepharose resin was close to that for the wild-type protein (1.4 M NaCl) except for the double mutant K128Q-K138Q which eluted at about 0.9 M NaCl. Caution should be exercised in comparing these data to that from the fluorescence quench experiments since elution of the protein from immobilized heparin with NaCl may not act solely to disrupt ionic interactions in the FGF–heparin complex, but may instead perturb the overall protein structure, resulting in a destabilization of the complex (see Discussion).

Receptor Binding. Heparin or HSPG interactions with FGF are required for the binding of FGF to its receptor (Yayon et al., 1991), and changes in the affinity of FGF for heparin are expected to be reflected in a diminished receptor binding affinity. Interaction of the mutant proteins with cell surface receptors was analyzed by measuring the ability of the mutant protein to compete with the binding of ^{125}I-labeled FGF-2 to cell surface receptors present on BHK-21 cells. As shown in Figure 2, no significant difference was observed for mutations involving residues 128 and 138 at site 1 as compared to the wild-type protein. Similar results were obtained for the site 2 mutants, R129 and K134 (data not shown). Although the mutant K128Q-K138Q exhibited a diminished affinity for heparin, this had no apparent effect on the receptor binding properties of the mutant. We reasoned, therefore, that the

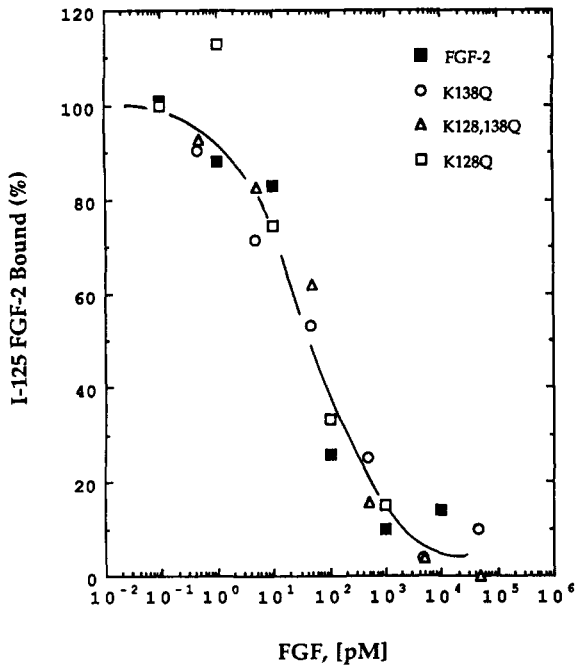

FIGURE 2: Competition by unlabeled FGF-2 and FGF-2 site 1 mutants (residues K128 and K138) for the binding of ^{125}I-FGF-2 to FGF receptors on BHK cells. Binding experiments were performed as described under Experimental Procedures. Binding of labeled FGF-2 to BHK cell surface receptors is expressed as percent of binding in the absence of unlabeled proteins. Nonspecific binding in the presence of a 100-fold excess of unlabeled FGF-2 did not exceed 10% of the total radioactivity bound. Each experimental point is the mean of duplicate determinations, and the error of the mean was less than 10%. The line drawn is for illustration purposes only.

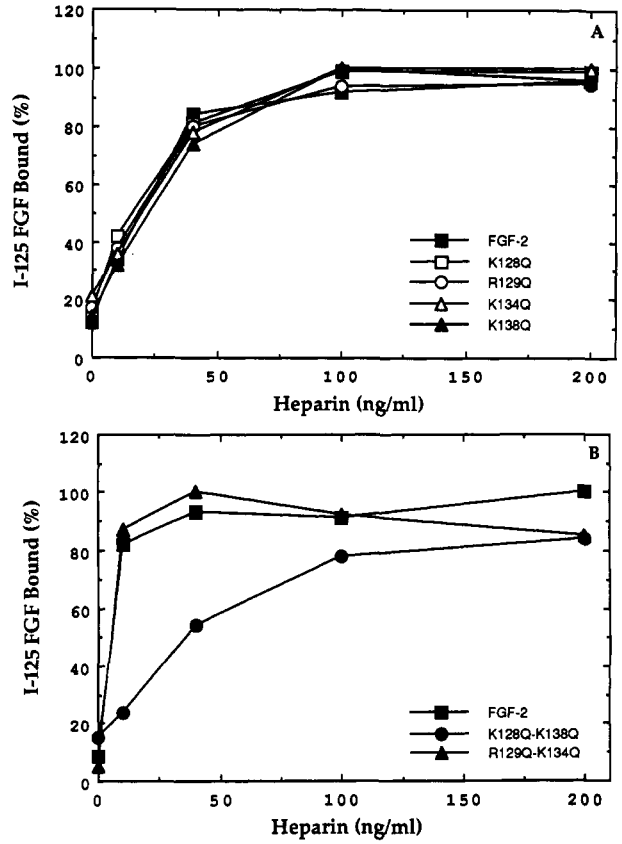

FIGURE 3: Effect of heparin on the binding of FGF-2 single-site mutants (panel A) and double-site mutants (panel B) to CHO 745 FGFR1 expressing cells. Binding was carried out in the presence of 5 ng/mL of ^{125}I-FGF-2 mutant, at 4 °C for 90 min, as a function of heparin concentration as described under Experimental Procedures.

lowered affinity of the protein for heparin may be compensated for by high local concentrations of HSPGs at the cell surface. Thus, we examined the receptor binding properties of the mutants using a Pgs A745 CHO cell line deficient in HSPG biosynthesis transfected with the fibroblast growth factor receptor type 1 (FGFR1) and followed receptor binding as a function of added heparin (Figure 3). As shown in Figure 3B only the K128Q-K138Q mutant exhibited diminished binding and required a 5–10-fold higher concentration of heparin to achieve the same level of binding observed for FGF-2 or the other mutants.

Endothelial Cell Proliferation and Heparin-Dependent DNA Synthesis in 32D-FGFR1 Myeloid Cells. The impact of the mutations on the activity of the FGF-2 proteins to induce proliferation of adult bovine aortic endothelial (ABAE) cells was determined as a function of added FGF in the culture media. The EC_{50} values for mutants K128Q and K128Q-K138Q in the site 1 group (Figure 4A) showed about a 10-fold increase, from about 0.5 to 5.0 ng/mL, whereas the mutant K138Q and all site 2 mutants (Figure 4B) showed no significant change as compared to the value for the wild-type protein. We next examined the dependence of DNA synthesis as a function of added heparin using 32D myeloid cells transfected with FGFR1. Myeloid cells do not express endogenous HSPGs and are, thus, a convienent cell type to investigate the effect of heparin on FGF biological activity by measuring the incorporation of [^{3}H]thymidine or increase in cell number. As shown in Figure 5, heparin at 100 ng/mL effectively promoted uptake of labeled thymidine in cells treated with the mutants or FGF-2 except for the K128Q-K138Q double mutant which was inactive. Stimulation of thymidine uptake was restored, however, to levels close to those for wild-type and other mutants at 500 ng/mL heparin. Thus, the increased dependence of K128Q-K138Q on heparin to induce DNA synthesis provides evidence that is consistent with a diminshed affinity of the protein for heparin. Of interest is that the K128Q mutant shown by the ABAE cell proliferation assay to be about 10-fold less active that FGF-2 (Figure 4B) was equipotent with wild-type FGF-2 in the heparin-dependent [^{3}H]thymidine uptake assay (Figure 5). The diminished activity of K128Q in the cell proliferation assay would appear to be unrelated to any direct effect on heparin–FGF interactions in view of its behavior in other FGF-specific assays.

Induction of PA Production. FGF-2 induces PA production in endothelial cells (Presta et al., 1989). The capacity of the mutant FGF proteins to induce plasminogen activator production in cultured ABAE cells was evaluated by determination of plasmin activity, which is proportional to cell-associated PA activity. The PA-inducing activity of the K128Q-K138Q mutant was dramatically reduced to a level about 10% that of the wild-type protein (Figure 6). The activities of the other mutants were close to that for FGF-2 except for R129Q-K134Q which showed a somewhat enhanced activity (Figure 6).

Induction of Capillary-like Tube Formation in Vitro. Cultured endothelial cells form tubular networks (Folkman & Haudenschild, 1980). Montesano et al. (1985) reported that FGF-2 induces capillary endothelial cells grown to confluency on three-dimensional collagen gels to invade the gel and form capillary-like tubular structures. This phenomenon is thought to mimic the formation of new blood vessels *in vivo*. Using this experimental model of *in vitro* angiogenesis, the mutants were evaluated further for their ability to induce the formation of capillary-like structures or tubes by ABAE cells cultured on three-dimensional collagen gels. The extent of the formation of tubes was quantitated

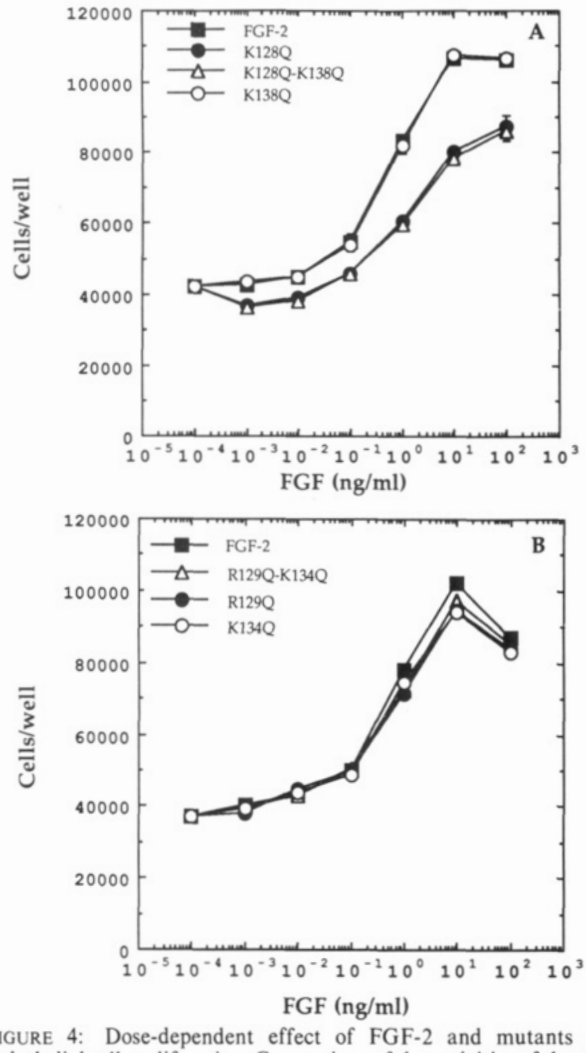

FIGURE 4: Dose-dependent effect of FGF-2 and mutants on endothelial cell proliferation. Comparison of the activities of the site 1 and site 2 mutants on the FGF-2-dependent proliferation of cultured ABAE cells was as described under Experimental Procedures. Panel A: Site 1 mutants, residues K128 and K138; Panel B: Site 2 mutants, residues R129 and K134.

using computer-assisted image analysis (Figure 7). Tube formation induced by the mutant K128Q-K138Q was much less than that induced by wild-type FGF-2 and other mutants. In view of the diminished PA-inducing activity of the K128Q-K138Q, this finding is consistent with the observation that induction of PA plays an important role in the formation of capillary-like tubes *in vitro* (Folkman & Shing, 1992).

DISCUSSION

Several attempts have been made to define domains in the primary sequence of FGF-2 that participate in FGF–heparin interactions. Baird et al. (1988) employing overlapping synthetic peptidic fragments of FGF-2 tentatively identified two domains (residues 33–77 and 112–155) involved in heparin and receptor binding. In another study, Seno et al. (1990) examined the mitogenic and heparin binding properties of a series of N- and C-terminally truncated forms of FGF-2 and concluded that heparin binding was confined to the carboxy-terminal third of the molecule. Using a different approach, Heath et al. (1991) examined the impact of charge alteration in the three positively charged domains in FGF-2 (residues 27–31, 116–119, and 128–138) and achieved only modest changes in the heparin-dependent mitogenic activity of a series of point mutants. Reductive methylation of K133 (Harper

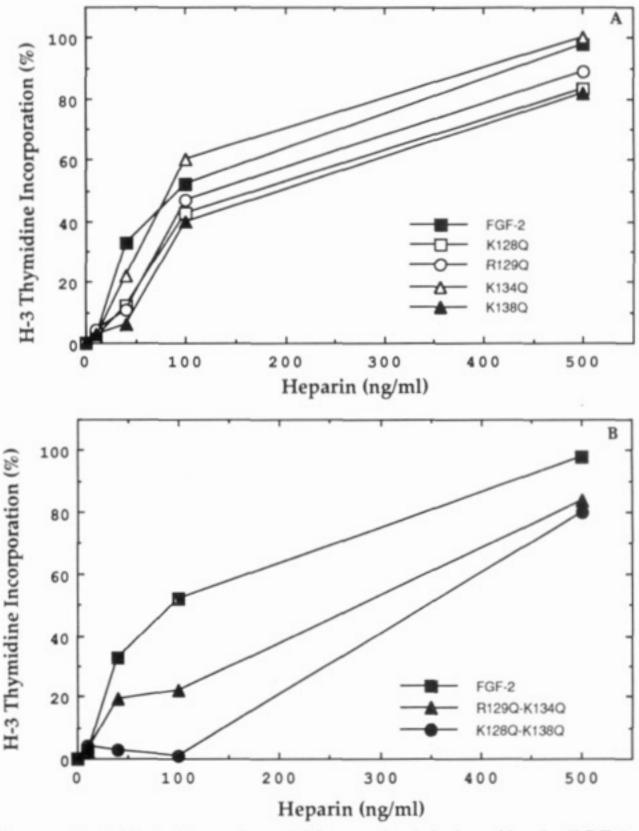

FIGURE 5: Effect of heparin on DNA synthesis induced by the FGF-2 single-site (panel A) and double-site mutants (panel B). [^{3}H]-Thymidine incorporation into 32D-flg cells was determined 24 h after treatment with 20 ng/mL of FGF-2 mutants as a function of heparin concentration (see Experimental Procedures for details).

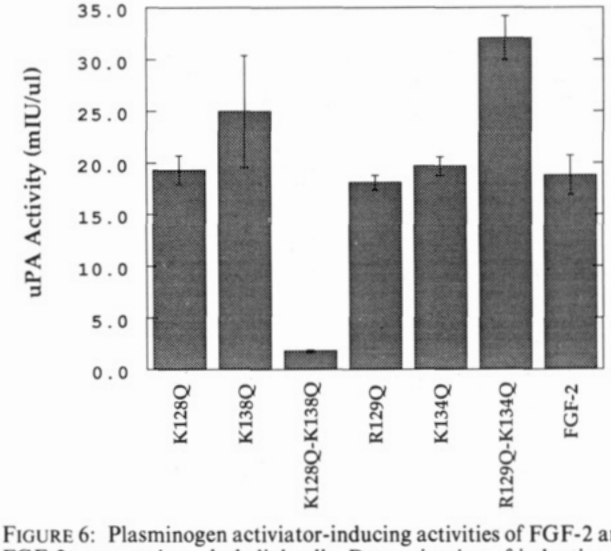

FIGURE 6: Plasminogen activiator-inducing activities of FGF-2 and FGF-2 mutants in endothelial cells. Determination of induction of cell-associated plasminogen activator after treatment of cells with 10 ng/mL FGF-2 or mutant protein was as described under Experimental Procedures.

& Lobb, 1988) in FGF-1 (equivalent to K134 in FGF-2) or substitution with glutamic acid (Burgess et al., 1990) led to a marked reduction in heparin affinity and biological properties. Evidence to support that the high affinity of FGF for heparin critically depends on an intact three-dimensional structure came from examination of the properties of two fragments of FGF-2, 27–69 and 70–153, obtained by exhaustive proteolysis of FGF-2 bound to heparin–Sepharose (Seddon et al., 1991). After elution from heparin Sepharose with 3 M NaCl the two fragments remain noncovalently associated

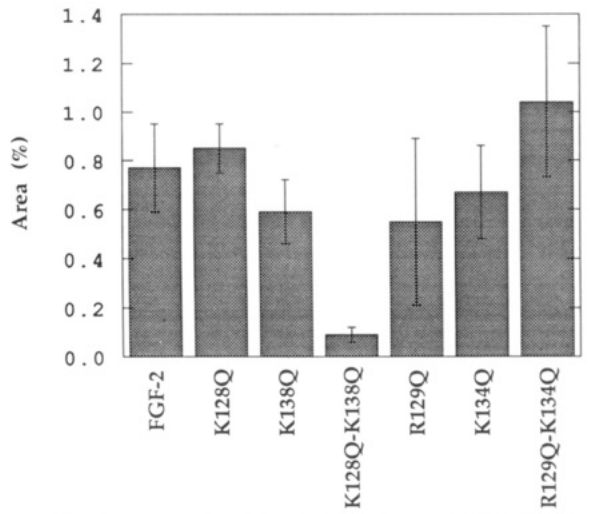

FIGURE 7: *In vitro* angiogenic activities of mutant FGF-2 proteins. The ability of FGF-2 mutants (10 ng/mL) to induce formation of tube-like structures by ABAE cells cultured on type I collagen gel was determined as described under Experimental Procedures. Data were collected from duplicate wells. Three to four areas of each well were examined by image analysis, and the mean value and standard deviation are presented.

and exhibit essentially the same properties as the intact protein. Denaturation of the complex resulted in dissociation of the fragments and loss of heparin binding properties; however, a significant residual mitogenic activity was found to be associated with the C-terminal fragment. Furthermore, the X-ray crystal structure of FGF-2 revealed that two pairs of basic residues, K128 and K138, and R129 and K134, respectively, formed ionic interactions with two sulfate ions (Eriksson et al., 1991; Zhang et al., 1991) and that these interactions may mimic those of the protein and sulfate moieties of heparin. Thus, evidence derived from different experimental approaches strongly favors the C-terminus of FGF and particularly the sequence 128–138 to be a heparin binding domain and that high-affinity binding to heparin is dependent on an intact tertiary structure.

In the present study the contributions of the positively charged side chains K128, R129, K134, and K138 in the molecular recognition of heparin by FGF-2 were investigated. Single-residue or double-residue replacements with glutamine at R129 and K134 had no significant impact on the biological properties compared to wild-type FGF. Single replacements at K128 and K138 gave a 10-fold decrease in heparin binding, but no significant change in receptor binding or biological properties with the exception of K128Q in the cell proliferation assay. Although up to a 100-fold change in the K_D for heparin was observed in this series of mutants, the decrease in affinity was not mirrored by significant changes in the molarity of NaCl required to elute the proteins from heparin affinity resins, except for the K128Q-K138Q mutant (see below). Consistent with the lowered heparin affinity of the K128Q-K138Q mutant is the significant reduction in biological activity compared to the wild-type protein. Of interest is that the diminished heparin binding capacity of K128Q-K138Q did not affect binding to cell surface receptors on cultured BHK cells, suggesting that the lower affinity toward heparin of the mutant may be overcome by the high local concentrations of HSPGs at the cell surface. To address this, binding of the K128Q-K138Q and other mutants to HSPG-deficient CHO cells was examined. In accord with the role of heparin in receptor binding the lowered heparin binding affinity of the K128Q-K138Q mutant resulted in an increased dependence on heparin to promote receptor–FGF interactions (Figure 3). In agreement with this observation is the increased heparin dependence for induction of DNA synthesis in 32D-FGFR1 myeloid cells by the K128Q-K138Q mutant (Figure 5). Although high concentrations of HSPG at the cell surface apparently compensate for the lowered heparin binding of the K128Q-K138Q mutant, it is not sufficient to enable the mutant protein to induce FGF-2-like activities, such as production of plasminogen activator and cell proliferation. In addition, we found that K128Q-K138Q when tested in an *in vitro* angiogenesis assay using cultured ABAE cells exhibited a diminished capacity to induce formation of capillary-like tube structures compared to FGF-2 and the other mutants, which is consistent with the proposed role of plasminogen activator in neovascularization (Folkman & Shing, 1992). Of note is that in cell proliferation assays the activity of the K128Q mutant was similar to that of K128Q-K138Q, whereas in other assays an FGF-2-like activity profile was maintained. Since the cell proliferation assay is a relatively long-term assay (>48 h), in comparison to other assays used here, the lowered potency of the K128Q mutant may be related to its stability in culture or after internalization by the cells.

Some of the properties of K128Q-K138Q mutant are similar to an FGF-2 mutant, M6B-FGF, described by Presta et al. (1992), in which residues K118, K119, K128, and R129 were simultaneously replaced with glutamine. M6B-FGF exhibited a diminished affinity for heparin (eluting from immobilized heparin at 0.95 M NaCl compared to 1.1 M NaCl for the wild-type protein), but identical receptor binding properties to wild-type FGF-2 in binding to cell surface high-affinity receptors. The ability of M6B-FGF to induce endothelial cell proliferation in culture was indistinguishable from that of the wild-type protein. On the other hand, the mutant showed a reduced ability to induce urokinase production by cultured endothelial cells and chemotactic activity. Consistent with the altered *in vitro* properties of the molecule, M6B-FGF lacked a significant angiogenic activity *in vivo*.

Cardin and Weintraub (1989) proposed consensus sequences for identification of heparin binding regions in proteins based on the organization of basic and hydropathic residues in the heparin binding domains of four heparin binding proteins. The consensus sequences [X-B-B-X-B-X] and [X-B-B-B-X-X-B-X], where B is the probability of occurrence of a basic residue and X is a hydropathic residue, predict two potential heparin binding regions in the human sequence of FGF-1 (residues 21–28 and 114–120) and one site in FGF-2 (residues 29–36). In view of the evidence from mutagenesis studies and X-ray crystallographic data, the use of such consensus sequences, at least for FGF-1 and -2, is not predictive of heparin binding regions. Rather, heparin binding domains in FGF are defined conformationally and not by a linear sequence. Recently, the crystal structure of a 1:1 complex between FGF-1 and the antiulcer drug sucrose octasulfate (SOS) was reported (Zhu et al., 1993). Residues of FGF-1 with side chains interacting with SOS are K127, K133, and R137. These residues are equivalent to K128, K134, and K138 in FGF-2. Interestingly, the structure indicates that K128 (R129 in FGF-2), in contrast to the unliganded structure, is disordered in the complex. In addition to positively charged residues in the FGF-1–SOS complex, the side chains of N33, N129, and Q142 form hydrogen bonds to the SOS sulfate moieties (corresponding to N34, T130, and Q143, respectively, in FGF-2). Of note is that the interaction of SOS or BCDS with FGF-2 is not sufficient to promote binding of FGF to its receptor (A. Yayon et al., unpublished data). This finding

is consistent with the observation that oligosaccharides of equal to or greater than 8–10 units in length are required for receptor binding of FGF-2 (Aviezer et al., 1994), suggesting that sites other than those shown to interact with SOS, and in the present study, are involved in heparin binding. In the light of X-ray crystallographic data one would expect that neutralization of any one of the positively charged side chains of K128, R129, K134, and K138 in FGF-2 would impact dramatically on high-affinity heparin binding. This, however, was not the case and was only observed for mutations at K128 and K138, indicating that these positions are particularly sensitive to disruption of salt bridges established between lysine side chains and the sulfate moieties of heparin. Several explanations for the behavior of the mutant proteins may be envisioned. The loss of arginine and lysine side chain–sulfate ion pairs may be partially compensated for by the formation of hydrogen bond interactions between the amide side chain of the introduced glutamine residue and the negatively charged sulfate moieties of heparin. Furthermore, neutralization of positive charge may be offset by relief of destabilizing repulsive electrostatic forces. Alternatively, partial neutralization of this localized region of charge in FGF may promote other protein–heparin interactions by recruitment of other surface-exposed residues as suggested by molecular dynamics studies docking FGF and mutant proteins with defined heparin fragments (D. Kitchen and A. P. Seddon, unpublished data). Our data indicate that the contribution of salt bridge-type interactions to heparin binding is most important for residues K128 and K138 and that other types of interactions such as hydrogen bonding, Van der Waals potentials, and hydrophobic interactions may significantly contribute to binding. Mutations at positions R129 and K134 resulted in a somewhat higher affinity of the protein toward heparin for the single-site replacements and no significant change for the double mutant compared to wild-type FGF-2 (Table 1), which perhaps best illustrates the importance of interactions other than salt bridge formation.

During the preparation of this paper for publication, Thompson et al. (1994) described the energetic characterization of FGF-2–heparin interactions and the contribution of residues K128, R129, K134, and K138 and identified additional potential contact points for heparin binding. In agreement with our studies, the authors conclude that the heparin binding domain is a discontinuous epitope and that specific nonionic interactions contribute about 70% of the free energy of binding and confirm participation of residues K128, R129, K134, and K138 in binding to heparin. Although qualitatively similar, our data differ somewhat from that presented by Thompson et al. (1994). Residues identified as potential heparin contact points were replaced by alanine in the study by Thompson et al., whereas we used glutamine. Lysine or arginine replacements with glutamine clearly implicate K128 and K138 as being the most important residues involved in heparin binding. Inspection of the $\Delta G°$ values presented in Table 1 reveals that K128 and K138 contribute about 24% of the total free energy of binding. When these residues were replaced with alanine (Thompson et al., 1994), however, the apparent contribution of the residues is reduced to about 16%. Using alanine, K134 [equivalent to K125 in the 146-residue numbering system for an N-terminally truncated form of FGF-2 used by Thompson et al. (1994)] emerges as the most important residue, providing about 20% of the free energy of binding, compared to K128 and K138, contributing only about 8% each. In the present study, K134 and also R129 were relatively insensitive to substitution by glutamine, either individually or simultaneously. That neutralization of charge at these positions tends to increase the affinity of the protein toward heparin (Table 1), suggesting that the contribution of salt bridge formation to binding is minimal. Thus, replacement of targeted residues with glutamine yields a different free energy binding profile than the alanine replacements used by Thompson et al. (1994). More importantly, a good correlation exists between the K_D values for the set of mutations using glutamine and the biological activities of the mutants. What is intriguing, when comparing the properties of the glutamine mutants to those of the alanine replacements, is that Thompson et al. were able to establish a linear relationship between the K_D's for heparin and molarity of NaCl required to elute the proteins from heparin–Sepharose. It was not possible to construct a similar relationship for our set of glutamine-containing mutants. It should be noted that the NaCl concentration-dependent elution of proteins from an affinity ligand-based resin may not be a direct assessment of the interactions between the protein and the ligand, but rather a measurement of a combination of effects that result from perturbation of the structural integrity of the protein and subsequent disruption of the forces of interactions, both of which are induced by the often high salt concentrations used to elute the proteins from affinity-based resins. For the case where glutamine is used to replace lysine or arginine in FGF-2 and NaCl is used to disrupt the heparin–Sepharose–FGF complex, little or no change in the concentration of NaCl required to elute the protein compared to the wild-type protein may be explained on the basis that both the FGF–chloride ion complex eluted from the heparin–Sepharose and the FGF–heparin–Sepharose complex is destabilized to a similar degree. When alanine is used, the FGF–heparin–Sepharose complex is destabilized relative to the FGF–chloride ion complex, and the difference is reflected by a decrease in the molarity of NaCl required to elute the protein from the resin.

The mutants in this study highlight the region where heparin binds and reveal that simple electrostatic arguments are insufficient to account for the interaction of FGF with heparin. The significance of FGF–heparin (HSPG) interactions, both extracellularly at the level of the receptor and intracellularly, dictates a detailed understanding of the binding interactions between FGF and FGF receptors with HSPG species to aid in the design of new therapeutic agents that modulate protein–HSPG interactions.

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REFERENCES

Aviezer, D., Levy, E., Safran, M., Svahn, C., Buddecke, E., Schmidt, A., David, G., Vlodavsky, I., & Yayon, A. (1994) *J. Biol. Chem. 269*, 114–121.

Baird, A., & Böhlen, P. (1990) in *Peptide Growth Factors and their Receptors* (Sporn, M., & Roberts, A., Eds.) *Handbook of Experimental Pharmacology*, Vol. 95 (1), pp 369–418, Springer-Verlag, New York.

Baird, A., Schubert, D., Ling, N., & Guillemin R. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 2324–2328.

Basilico, C., & Moscatelli, D. (1992) *Adv. Cancer Res. 59*, 115–165.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–255.

Burgess, W. H., Shaheen, A. M., Ravera, M., Jaye, M., Donohue, P. J., & Winkles, J. A. (1990) *J. Cell Biol. 111*, 2129–2138.
Cardin, A. D., & Weintraub, H. J. R. (1989) *Atheriosclerosis 9*, 21–32.
Eriksson, E. A., Cousens, L. S., Weaver, L. H., & Matthews, B. W. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 3441–3445.
Esko, J. D. (1991) *Curr. Opin. Cell. Biol. 3*, 805–816.
Folkman, J., & Haudenschild, C. (1980) *Nature 288*, 551–556.
Folkman, J., & Shing, Y. (1992) *J. Biol. Chem. 267*, 10931–10934.
Gospodarowicz, D., & Cheng, J. (1986) *J. Cell. Physiol. 128*, 475–484.
Gospodarowicz, D., Cheng, J., Lui, G. M., Baird, A., & Böhlen, P. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 6963–6967.
Guimond, S., Maccaranna, M., Olwin, B. B., Lindahl, U., & Rapraeger, A. C. (1993) *J. Biol. Chem. 268*, 23906–23914.
Habuchi, H., Suzuki, S., Saito, T., Tamura, T., Harada, T., Yoshida, K., & Kimata, K. (1992) *Biochem. J. 285*, 805–813.
Harper, J. W., & Lobb, R. R. (1988) *Biochemistry 27*, 671–678.
Heath, W. F., Cantrell, A. M., Mayne, N. G., & Jaskunas, S. R. (1991) *Biochemistry 30*, 5608–5615.
Ishai-Michaeli, R., Svahn, C. M., Weber, M., Chajek-Shaul, T., Korner, G., Ekre, H.-P., & Vlodavsky, I. (1992) *Biochemistry 31*, 2080–2088.
Ishihara, M., Tyrrell, D. J., Stauber, G. B., Brown, S., Cousens, I. S., & Stack, R. J. (1993) *J. Biol. Chem. 268*, 4675–4683.
Kjellén, L., & Lindahl, U. (1991) *Annu. Rev. Biochem. 60*, 443–475.
Lindahl, U. (1989) in *Heparin* (Lane, D. A., & Lindahl, U., Eds.) pp 115–190, CRC Press, Boca Raton, FL.
Maccarana, M., Casu, B., & Lindahl, U. (1993) *J. Biol. Chem. 268*, 23898–23905.
Mach, H., Volkin, D. B., Burke, C. J., Middauph, C. R., Linhardt, R. J., Fromm, J. R., Loganathan, D., & Mattson, L. (1993) *Biochemistry 32*, 5480–5489.
Mali, M., Elenius, K., Miettinen, H. M., & Jalkanen, M. (1993) *J. Biol. Chem. 268*, 24215–24222.
Miyamoto, M., Naruo, K.-I., Seko, C., Matsumoto, S., Kondo, T., & Kurokawa, T. (1993) *Mol. Cell. Biol. 13*, 4251–4259.
Montesano, R., & Orci, L. (1985) *Cell 42*, 469–477.
Moscatelli, D. (1987) *J. Cell. Physiol. 131*, 123–130.
Nathan, C., & Sporn, M. (1991) *J. Cell Biol. 113*, 981–986.
Nugent, M. A., & Edelman, E. R. (1992) *Biochemistry 31*, 8876–8883.
Nurcombe, V., Ford, M. D., Wildschut, J. A., & Bartlett, P. F. (1993) *Science 260*, 103–106.
Presta, M., Maier, J. A. M., & Ragnotti, G. (1989) *J. Cell Biol. 109*, 1877–1884.
Presta, M., Statuto, M., Isacchi, P., Caccia, P., Pozzi, A., Gualandris, A., Rusnati, M., Bergonzoni, L., & Sarmientos, P. (1992) *Biochem. Biophys. Res. Commun. 185*, 1098–1107.
Reiland, J., & Rapraeger, A. C. (1993) *J. Cell. Sci. 105*, 1085–1093.
Roghani, M., & Moscatelli, D. (1992) *J. Biol. Chem. 267*, 22156–22162.
Rosenberg, A. H., Lade, B. N., Chui, D., Dunn, J. J., & Studier, F. W. (1987) *Gene 56*, 125–135.
Ruoslahti, E., & Yamaguchi, Y. (1991) *Cell 64*, 867–869.
Seddon, A. P., Decker, M., Muller, T., Armellino, D., Kovesdi, I., Gluzman, Y., & Böhlen, P. (1991) *Ann. N.Y. Acad. Sci. 638*, 98–108.
Seno, M., Sasada, R., Kurokawa, T., & Igarashi, K. (1990) *Eur. J. Biochem. 188*, 239–245.
Sommer, A., & Rifkin, D. B. (1989) *J. Cell. Physiol. 138*, 215–220.
Taira, K., & Benkovic, S. J. (1987) *J. Med. Chem. 31*, 129–137.
Thompson, L. D., Pantoliano, M. W., & Springer, B. A. (1994) *Biochemistry 33*, 3831–3840.
Turnbull, J. E., Fernig, D., Ke, Y., Wilkinson, M. C., & Gallagher, J. T. (1992) *J. Biol. Chem. 267*, 10337–10341.
Vlodavsky, I., Folkman, J., Sullivan, R., Fridman, R., Ishai-Michaeli, R., Sasse, J., & Klagsbrun, M. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 2292–2296.
Volkin, D. B., Tsai, P. K., Dabora, J. M., Gress, J. O., Burke, C. J., Linhardt, R. J., & Middaugh, C. R. (1993) *Arch. Biochem. Biophys. 300*, 30–41.
Walker, A., Turnbull, J. E., & Gallagher, J. T. (1994) *J. Biol. Chem. 269*, 931–935.
Yanagishita, M., & Hascall, V. C. (1992) *J. Biol. Chem. 267*, 9451–9454.
Yayon, A., Klagsbrun, M., Esko, J. D., Leder, P., & Ornitz, D. M. (1991) *Cell 64*, 841–848.
Zhang, J., Cousens, L. S., Barr, P., & Sprang, S. R. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 3446–3450.
Zhu, X., Komiya, H., Chirino, A., Faham, S., Fox, G. M., Arakawa, T., Hsu, B. T., & Rees, D. G. (1991) *Science 251*, 90–93.
Zhu, X., Hsu, B. T., & Rees, D. C. (1993) *Structure 1*, 27–34.