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Mutations in the Heparin-Binding Domains of Human Basic Fibroblast Growth Factor Alter Its Biological Activity

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ABSTRACT: Eleven structural analogues of human basic fibroblast growth factor (bFGF) have been prepared by site-directed mutagenesis of a synthetic bFGF gene to examine the effect of amino acid substitutions in the three putative heparin-binding domains on FGF's biological activity. After expression in Escherichia coli, the mutant proteins were purified to homogeneity by use of heparin–Sepharose chromatography and analyzed for their ability to stimulate DNA synthesis in human foreskin fibroblasts. Recombinant human bFGF 1-146 and [Ala^{69},Ser^{87}]bFGF, an analogue where two of the four cysteines had been replaced by alanine and serine, were equipotent to standard bovine basic fibroblast growth factor. Substitution of aspartic acid-19 by arginine in the first heparin-binding domain yielded a molecule that stimulated a higher total mitogenic response in fibroblasts as compared to bFGF. In addition, replacement of either arginine-107 in the second domain or glutamine-123 in the third domain with glutamic acid resulted in compounds that were 2 and 4 times more potent than bFGF. In contrast, substitution of arginine-107 with isoleucine reduced the activity of the molecule by 100-fold. Combination of domain substitutions to generate the [Glu^{107,123}]bFGF and [Arg^{19},Lys^{123,126}]bFGF mutants did not show any additivity of the mutations on biological activity. Alterations in the biological activity of the analogues was dependent on both the site of and the type of modification. Increased positive charge in the first domain and increased negative charge in the second and third domains enhanced biological potency. The altered activities of the derivatives appear to be due in part to changes in the affinity of the analogues for heparin. We conclude that changes in all three of the putative heparin–binding domains result in altered mitogenic activity and heparin interaction of basic fibroblast growth factor.

Basic fibroblast growth factor is a member of a family of heparin-binding polypeptide growth factors that have been shown to be potent mitogens for a wide variety of cells of mesodermal and neuroectodermal origin (Gospodarowicz et al., 1987). In addition, basic FGF[1] has been shown to be a potent stimulator of angiogenesis in vivo (Gospodarowicz et al., 1979, 1984; Esch et al., 1985) and to stimulate collagenase and plasminogen activator secretion, chemotaxis, and mitogenesis in capillary endothelial cells in vitro (Presta et al., 1986). The discovery of the strong affinity of FGF for heparin (Shing et al., 1984) has allowed the isolation and characterization of a number of endothelial cell mitogens of 13 000–18 000 molecular weight with a strong affinity for heparin and a basic isoelectric point (p*I*) (Lobb et al., 1986). Further work has shown that these factors are all forms of basic FGF differing in their N-terminal sequences as a result of cleavage by acid proteases (Klagsbrun et al., 1987). The significance of this amino-terminal heterogeneity is not known. Pituitary-derived basic fibroblast growth factor has been shown to be a single chain protein consisting of 146 amino acids (Esch

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[1] Abbreviations: aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; DME, Dulbecco's-modified Eagle's medium; ED_{50}, half-maximal stimulatory dose; FBS, fetal bovine serum; FGF, fibroblast growth factor; rbFGF, recombinant Met-Arg-Leu-[Ala^{69},Ser^{87}]bFGF; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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et al., 1985). Isolation and characterization of cDNA clones for the bovine and human basic FGFs predict a translation product of 155 amino acids with no obvious signal peptide (Abraham et al., 1986a,b). Forms of basic FGF longer than 155 amino acids have been observed (Moscatelli et al., 1987; Sommer et al., 1987, 1989) and appear to arise from alternative translation initiation sites using CUG codons (Prats et al., 1989; Florkiewicz & Sommer, 1989).

There are currently six other known members of the heparin-binding growth factor family. Acidic fibroblast growth factor (aFGF) exists in multiple forms almost exclusively in neural tissues (Lobb et al., 1986). The amino acid sequence of acidic FGF reveals a 140 amino acid protein with 55% homology to basic FGF (Gimenez-Gallego, 1985). Like basic FGF, the acidic FGF cDNA, the product of a different gene, codes for a 155 amino acid precursor that also lacks a traditional signal sequence and is apparently not secreted (Jaye et al., 1986). In contrast, four out of the five other members of the FGF family appear to be secreted glycoproteins that display 40–50% homology with basic and acidic FGFs. These are the hst/KFGF oncogene (Delli Bovi et al., 1987; Taira et al., 1987), the int-2 gene product (Smith et al., 1988), the FGF-5 oncogene (Zhan et al., 1988), the FGF-6 oncogene (Marics et al., 1989), and the keratinocyte growth factor (FGF-7) (Finch et al., 1989). While these other members of the FGF family are not well characterized, they appear to share many of the properties of acidic and basic FGF, such as the ability to bind heparin and to stimulate fibroblast and endothelial cell mitogenesis.

The multiplicity of heparin-binding growth factors raises questions about the physiological role of such proteins. In addition, the lack of signal peptides in the precursors of acidic and basic fibroblast growth factor makes it unclear how such factors are released from their source of synthesis to surrounding tissues. However, the widespread prevalence of these two proteins and their receptor(s) points to some mode of release and utilization (Neufield & Gospodarowicz, 1985; Olwin & Hauschka, 1989). Fibroblast growth factor has been shown to be deposited in the extracellular matrix of the basement membrane of cells both in vitro and in vivo and can be released in a biologically active form (Baird & Ling, 1987; Bashkin et al., 1989; Folkman et al., 1988; Moscatelli, 1987, 1988; Presta et al., 1989; Vigny et al., 1988; Vlodavsky et al., 1987). The growth factor is bound to heparan sulfate proteoglycan in the extracellular matrix (Bashkin et al., 1989; Folkman et al., 1988; Vigny et al., 1988), but the route by which this takes place is unknown.

This strong affinity for heparin also complicates the analysis of the FGF signaling pathway. Both acidic and basic FGF appear to react with a common receptor of molecular mass 125–145 kilodaltons on the cell surface (Neufield & Gospodarowicz, 1986). The affinity of FGF binding to cells has been reported to range from 8 pM to 30 nM (Olwin & Hauschka, 1989). This disparity in values appears to have an explanation in FGF's ability to interact with heparin, including cell-surface-bound heparin, leading to the interpretation of low- and high-affinity cell-surface receptors. The high-affinity cell-surface receptor has recently been cloned and reveals an apparent extracellular domain connected to an intracellular tyrosine kinase domain (Isacchi et al., 1990; Lee et al., 1989; Reid et al., 1990), placing FGF in a larger family of growth factors with tyrosine kinase containing receptors. The low-affinity binding sites comprise the majority of FGF binding to the cell (up to 90%) and are the result of FGF interaction with sulfated glycosaminoglycans (Kan et al., 1988; Moscatelli, 1987, 1988). This heparin-bound population of FGF serves as a reservoir of FGF activity and appears to be important to FGF's biological actions (Flaumenhaft et al., 1989; Moscatelli, 1987, 1988; Presta et al., 1989).

In addition to regulating FGF's interaction with cells, heparin has also been shown to play an important role in protecting the molecule from inactivation by proteases and extremes of pH (Damon et al., 1989; Gospodarowicz & Cheng, 1986; Lobb, 1988; Rosengart et al., 1988; Saksela et al., 1988). However, FGF complexed to heparin and to heparan sulfate proteoglycan is as active as free FGF (Moscatelli, 1987, 1988; Saksela et al., 1988). While some studies have demonstrated that different portions of the FGF molecule are involved in receptor- and heparin-binding (Baird et al., 1988; Kurokawa et al., 1989; Schubert et al., 1987), others have demonstrated that the ability of FGF to bind heparin is important for biological activity (Harper & Lobb, 1988; Lobb, 1988).

Given the recent demonstrations that basic fibroblast growth factor can be produced by recombinant means (Barr et al., 1988; Iwane et al., 1987; Squires et al., 1988), we decided to address the relationship of heparin-binding affinity to biological activity with a series of site-directed mutants. Three regions of the basic FGF molecule have been identified as being potential heparin-binding sites on the basis of clusters of basic amino acids in the sequence and limited structure-function studies (residues 18–22, 107–110, and 119–129) (Baird et al., 1988; Esch et al., 1985; Gospodarowicz et al., 1987; Harper & Lobb, 1988; Lobb, 1988; Sommer et al., 1987). We introduced residues into the three putative heparin-binding regions of human basic FGF that had the effect of changing the net charge of these areas and investigated the biological activity of the resulting mutants. Alterations in the heparin-binding domains of the FGF molecule had subtle but significant effects on mitogenic ability that correlated with the site and type of charge change.

Materials and Methods

Materials. Recombinant bovine basic fibroblast growth factor and human platelet-derived growth factor were obtained from Amgen. Bovine acidic fibroblast growth factor was purchased from R & D Systems while murine epidermal growth factor was obtained from Collaborative Research. Human insulin-like growth factors I and II were generously provided by Drs. Richard DiMarchi and Michele Smith, Lilly Research Laboratories. Porcine intestinal, bovine lung, and low molecular weight heparins were purchased from Sigma and Calbiochem.

Gene Construction and Expression. Analogues of bFGF were constructed via oligonucleotide cassette mutagenesis of a synthetic human bFGF gene.[2] All synthetic bFGF gene sequences were verified by the chain termination method of Sanger et al. (1977) prior to expression. To facilitate expression of the mutant bFGF derivatives, they were transferred to a pBR322-based expression vector that contains the bacteriophage λ P_L promoter under the control of the thermal CI857 repressor (Figure 1A). The mutant expression plasmids were transformed into *Escherichia coli* L201 [htpR165 *lonR9 supC*^{ts}; derived from LC137 (Goff & Goldberg, 1985)], cultures were grown to an OD_{600} of 0.4 in 2× tryptone yeast medium, and FGF gene expression was induced by raising the temperature to 42°C.

Purification of FGF from E. coli Cells. FGF-containing granules were isolated from 5–7 g (wet weight) of *E. coli* in

[2] DNA sequence of the synthetic bFGF gene is available upon request to the corresponding author.

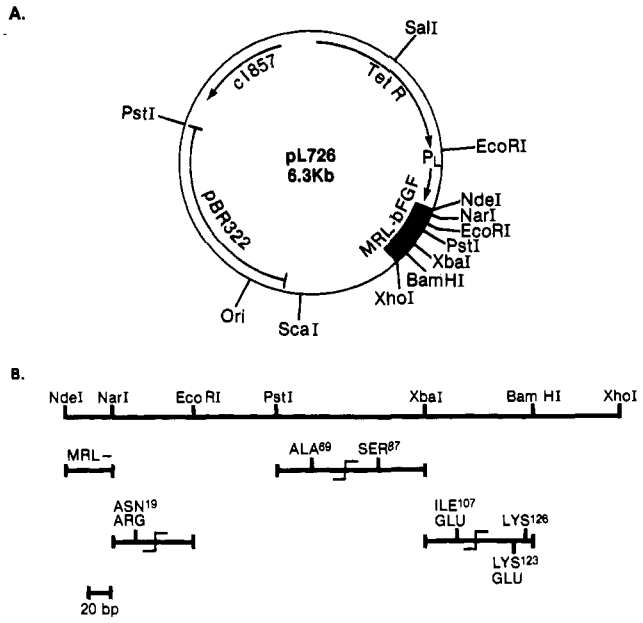

FIGURE 1: (A) Map of the Met-Arg-Leu-bFGF expression vector (pL726). Relative positions of the λP_L promoter, the CI857 repressor gene, the tetracycline resistance gene (TetR), the region of the plasmid derived from pBR322, the origin of replication (ori), and the restriction sites within the bFGF gene pertinent to analogue construction are shown. Arrows indicate the direction of transcription. (B) Schematic diagram depicting the bFGF cassette mutagenesis strategy. The bars represent regions of the gene replaced by synthetic oligonucleotide linkers. Splice points of tandem linkers are indicated by (⌐) symbols. The locations of the substituted amino acids are shown.

accordance with standard procedures (Iwane et al., 1987; Seno et al., 1988). The isolated granule pellet was solubilized in 20 mM Tris-HCl/6 M guanidine hydrochloride/0.2 M dithiothreitol, pH 8.5, with stirring at 22 °C for 12 h. The solubilized material was slowly diluted with 20 mM Tris-HCl, pH 7.2, to a final chloride concentration of 0.2 M, and the resulting solution was stirred for 2 h to allow for protein renaturation. The FGF analogues were purified on a 1.6 × 12 cm column of heparin–Sepharose (Pharmacia LKB Biotechnology, Inc.) eluted with a 7-h linear NaCl gradient of 0–3.0 M in 20 mM Tris-HCl, pH 7.2, 1.5 mL/min. Fractions containing FGF activity were pooled and stored at −20 °C until further use.

Mitogenesis Assays. Human foreskin fibroblasts (a gift from Dr. Lawrence Slieker, Lilly Research Laboratories) were grown to confluence in 24-well cell plates (Costar) with use of Dulbecco's-modified Eagle's medium (DME) supplemented with 10% fetal bovine serum (FBS) (Hyclone). The medium was then switched to DME/0.2% FBS (1 mL/well), and the cells were incubated for 4 days to allow them to become quiescent. Then, 0.2 mL of DME/0.2% FBS containing 1 μCi of [^{3}H]thymidine (6.7 Ci/mmol)(New England Nuclear) and mitogen or control were added to each well. The plates were incubated for 48 h at 37 °C, and thymidine incorporation was measured. All assay points were run in triplicate, and each analogue was tested in a minimum of five separate experiments. ED_{50} values for all compounds were determined from the dose necessary to reach half the maximum stimulation of thymidine incorporation by basic FGF in that experiment. The results shown in Figures 3–5 contain data from multiple experiments and represent the average mitogenic response of each analogue.

Other Methods. Protein determination was done by the method of Bradford (1976) and by UV absorbance at 278 nm with an extinction coefficient of 1.3 for a 0.1% FGF solution (Fox et al., 1988). Gel electrophoresis was performed under reducing conditions according to Laemmli (1970), with protein visualization by Coomassie Blue staining.

Results and Discussion

Site-directed mutagenesis is a powerful tool in the study of the relationship between protein structure and function. This study was initiated to probe the connection between heparin binding and biological activity of basic fibroblast growth factor.

Selection of FGF Analogues. The selection of alterations in the FGF sequence was based on two presumptions: (1) that the heparin-binding regions could be identified and (2) that alterations of the overall charge in the heparin-binding regions would change the molecule's affinity for heparin and the biological activity and/or stability. The three regions that were selected as heparin-binding domains were residues 18–22, 107–110, and 119–129, on the basis of previous studies (Baird et al., 1988; Esch et al., 1985; Gospodarowicz et al., 1987; Harper & Lobb, 1988; Lobb, 1988; Sommer et al., 1987) and an examination of homologous regions of the other members of the FGF family (Delli Bovi et al., 1987; Finch et al., 1989; Gimenez-Gallego et al., 1985; Marics et al., 1989; Smith et al., 1988; Taira et al., 1987; Zhan et al., 1988).

In this study, the cysteine residues at positions 69 and 87 in the FGF molecule were substituted with alanine and serine, respectively, to simplify purification and analysis (Arakawa et al., 1989; Fox et al., 1988; Seno et al., 1988). The FGF analogues can then be broken down into four groups. The first group involved substitution of aspartic acid-19 in the first heparin-binding domain with asparagine or arginine, which served to increase the net charge of the region by +1 and +2, respectively. The second group entailed the substitution of arginine-107 in the second heparin-binding domain with isoleucine or glutamic acid, changing the charge in this section by −1 and −2. The third group consisted of changes in the third heparin-binding domain. Glutamine-123 was substituted with glutamic acid, and the second analogue substituted lysine for both glutamine-123 and leucine-126. The fourth group corresponded to changes in two of the heparin-binding domains, i.e., Arg^{19} + $Lys^{123,126}$ and Glu^{107} + Glu^{123}, in order to analyze any additive effects of the single domain substitutions.

Gene Construction and Expression. The mutagenesis strategy is shown in Figure 1B. The Met-Arg-Leu leader peptide was chosen to enhance expression of basic FGF in *E. coli* in accordance with our previous observations that other small molecular weight proteins such as insulin and the insulin-like growth factors are expressed more efficiently in *E. coli* with Met-Arg at their 5′ termini (Cantrell et al., 1991). Since this strategy was found to give efficient expression of the desired protein and the biological activity of the recombinant Met-Arg-Leu-bFGF molecule was found to be indistinguishable from recombinant bovine bFGF (see below), no other expression systems were investigated. Next, the cysteine residues at positions 69 and 87 in the FGF molecule were substituted with alanine and serine, respectively, via a tandem *Pst*I/Xba*I* linker. All subsequent substitutions were made in this Met-Arg-Leu-[Ala^{69},Ser^{87}]bFGF construct.

Following mutagenesis, the FGF analogues were moved to a pBR322-based expression plasmid containing the λ P_L promoter under the control of the thermal-sensitive repressor CI857 (Figure 1A). All FGF analogues expressed at a level equal to that of recombinant Met-Arg-Leu-bFGF in a Lon protease deficient strain of *E. coli*. Figure 2 shows the SDS–PAGE analysis of induced *E. coli* L201 with and without the Met-Arg-Leu-bFGF plasmid. Attempts to express bFGF in *E. coli* strains with wild-type Lon were unsuccessful. Ap-

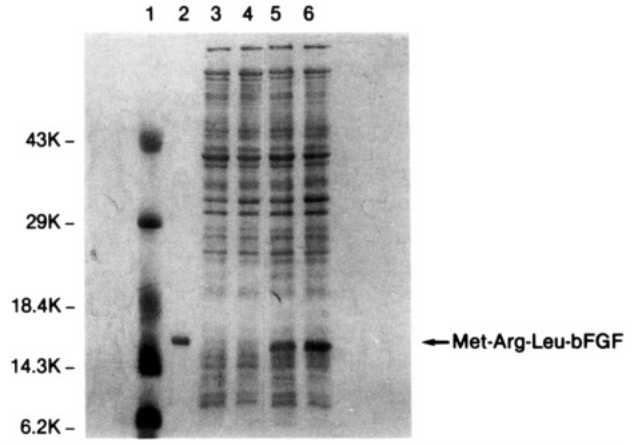

FIGURE 2: Expression of Met-Arg-Leu-bFGF in the *lon*⁻ strain of *E. coli*, L201. Samples were as follows: (1) Prestained low molecular weight markers, (2) recombinant bovine bFGF standard (0.2 μg), host cells without expression plasmid (3) 1 h after induction and (4) 3 h after induction, host cells containing the expression plasmid (pL726) (5) 1 h after induction and (6) 3 h after induction. The above samples were subjected to 15% SDS–PAGE under reducing conditions and stained with Coomassie Brilliant Blue.

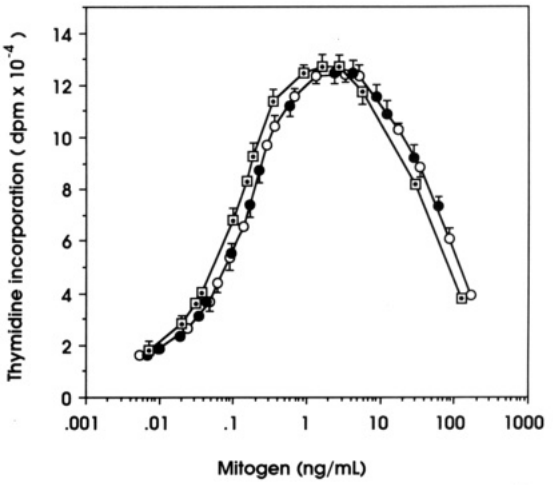

FIGURE 3: Mitogenic activity of recombinant FGF on human foreskin fibroblasts. The figure shows the dose response curves of recombinant bovine bFGF (○) versus recombinant Met-Arg-Leu-bFGF (●) and rbFGF (⊡). Assays were performed in triplicate and repeated at least five times.

parently, FGF, like other low molecular weight foreign proteins, is readily degraded by *E. coli* proteases. Expression of the analogues in a *lon*⁻ host strain increased their accumulation to levels detectable by Coomassie Blue staining.

Purification of FGF from E. coli Cells. After induction, the FGF analogue containing cells were isolated and lysed. No FGF protein could be detected in the soluble cell lysate fraction by gel electrophoresis or biological activity. Successful isolation of active FGF from the insoluble granules involved the use of denaturants and thiols followed by renaturation of the protein. Guanidine hydrochloride was found to be superior to urea in the solubilization process and was used throughout the study. Purification could be effected by a single heparin–Sepharose chromatography step to yield samples that showed a single band on SDS-PAGE gels (data not shown). Thiols were not included in the purification step since the removal of the two nonessential cysteine residues greatly reduces the formation of disulfide isomers (Fox et al., 1988; Seno et al., 1988). The yield of purified FGF analogue varied from 1 to 2 mg/g (wet weight) of *E. coli* cells. The various FGF analogues eluted from the heparin–Sepharose column at NaCl concentrations ranging from 1.4 to 2.0 M depending on the analogue. The elution patterns of the mutant proteins were found to be consistent with the charge changes that had been introduced. While recombinant Met-Arg-Leu-bFGF and Met-Arg-Leu-[Ala^{69},Ser^{87}]bFGF (rbFGF) eluted from heparin–Sepharose at 1.65 M NaCl, the more negative analogues containing glutamic acid at positions 107 and 123 eluted from the column at NaCl concentrations of 1.4–1.5 M. In contrast, the derivatives containing the substitutions Arg^{19} and $Lys^{123,126}$ were found to elute from the column at 1.8–2.0 M NaCl.

Biological Activities of Recombinant FGF Analogues. The purified FGF analogues were assayed for their ability to stimulate DNA synthesis in quiescent normal human foreskin fibroblasts. As shown in Figure 3, both recombinant Met-Arg-Leu-bFGF and rbFGF gave the same biphasic dose response curve as standard recombinant bovine bFGF. The biphasic dose response curve of fibroblast growth factors has been observed by other groups (Arakawa et al., 1989; Fox et al., 1988; Huang & Huang, 1986; Ke et al., 1990). The result in Figure 3 demonstrated the ability of our system to produce biologically active fibroblast growth factor and indicated that the Met-Arg-Leu leader peptide and the cysteine substitutions did not have a deleterious effect on activity.

In contrast, analogues with alterations in the heparin-binding domains did show significant differences in biological activity from recombinant bovine bFGF. These alterations fall into two categories: (1) analogues that vary from the native molecule in their potency on a dose response basis and (2) analogues that stimulated a higher total mitogenic response as compared to bFGF. This latter finding is significant because, in our hands, basic FGF was capable of stimulating at most a maximum response of a 10–15-fold increase in thymidine incorporation. No other growth factor or combination of growth factors studied (platelet-derived growth factor, acidic fibroblast growth factor, epidermal growth factor, insulin-like growth factors I and II) was able to surpass this level. Distinction on the basis of maximum mitogenic response has been seen before in studies comparing the growth potential of insulin molecules from various species. These studies showed that hystricomorph insulins are capable of giving a higher maximum response in growth-promoting assays as compared to pork and human insulins (King & Kahn, 1981; King et al., 1983). In the case of the FGF analogues discussed in this paper, differences in mitogenic activity most likely reflect (1) increased stabilization of the analogues against denaturation and/or proteolysis and (2) decreased sequestration of the analogues by the extracellular matrix due to altered affinities for heparin-like molecules resulting in increased levels of free mitogen in the assay (see below).

Substitution of Asp^{19} by Asn in the first heparin-binding domain resulted in a leftward shift in the dose response curve, while the Arg^{19} derivative showed a significant increase in total mitogenic response with little or no change in the ED_{50} value as compared to bFGF (Figure 4A and Table I). Asparagine represented an isosteric replacement that increased the net charge of the region by +1 and resulted in a distinct increase in biological activity. Arginine, on the other hand, increased the charge by +2 and substantially altered the potency of the molecule in that it produced a greater total mitogenic response. The altered biological activities of [Asn^{19}]rbFGF and [Arg^{19}]rbFGF were reflected in their chromatographic behavior of heparin–Sepharose, which showed increases in retention time over recombinant Met-Arg-Leu-bFGF. A recently published study reports on the preparation and characterization of a series of deletion mutants of basic FGF (Seno et al., 1990). Their data indicate that the amino-terminal

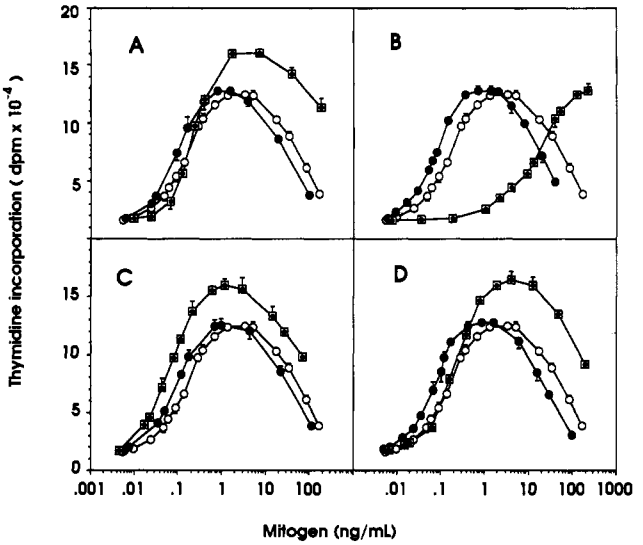

FIGURE 4: Effect of FGF analogues on DNA synthesis in quiescent human foreskin fibroblasts. The figure shows the dose response curves of recombinant bovine bFGF (○) versus FGF analogues substituted in the first heparin-binding domain, [Asn^{19}]rbFGF (●) and [Arg^{19}]rbFGF (⊡) (panel A); the second heparin-binding domain, [Glu^{107}]rbFGF (●) and [Ile^{107}]rbFGF (⊡) (panel B); the third heparin-binding domain, [$Lys^{123,126}$]rbFGF (●) and [Glu^{123}]rbFGF (⊡) (panel C); and multiple domains, [$Glu^{107,123}$]rbFGF (●) and [Arg^{19},$Lys^{123,126}$]rbFGF (⊡) (panel D).

Table I: Effect of Fibroblast Growth Factor Analogues on DNA Synthesis in Human Fibroblasts in the Presence and Absence of Soluble Heparin[a]

derivative	ED_{50} (ng/mL)	
	− heparin	+ heparin
recombinant bovine basic FGF	0.16 ± 0.05	0.20 ± 0.06
bovine acidic FGF	0.77 ± 0.14	0.22 ± 0.01
recombinant human Met-Arg-Leu-bFGF	0.15 ± 0.05	0.18 ± 0.04
rbFGF	0.12 ± 0.02	0.11 ± 0.01
[Asn^{19}]rbFGF	0.09 ± 0.01	0.08 ± 0.01
[Arg^{19}]rbFGF	0.16 ± 0.04	0.54 ± 0.01
[Glu^{107}]rbFGF	0.08 ± 0.03	0.08 ± 0.01
[Ile^{107}]rbFGF	12.60 ± 1.53	1.92 ± 0.11
[Glu^{123}]rbFGF	0.04 ± 0.01	0.07 ± 0.02
[$Lys^{123,126}$]rbFGF	0.11 ± 0.02	0.09 ± 0.01
[$Glu^{107,123}$]rbFGF	0.07 ■ 0.02	0.07 ± 0.02
[Arg^{19},$Lys^{123,126}$]rbFGF	0.14 ± 0.03	0.38 ± 0.01

[a] DNA synthesis was carried out as described in Materials and Methods in the presence and absence of 100 μg/mL heparin. Each value represents the average of at least four separate experiments that were performed in triplicate. ED_{50} values were determined from the dose of FGF analogue required to give half the maximum stimulation of thymidine incorporation by recombinant bovine basic FGF in the absence of heparin. rbFGF = recombinant Met-Arg-Leu-[Ala^{69},Ser^{87}]bFGF.

portion of the bFGF molecule is important for biological activity but is relatively unnecessary for heparin-binding. If correct, this finding raises the possibility that the increases in biological activity seen with the Asn^{19} and Arg^{19} FGF analogues are due to changes other than those due to heparin interaction and that the increased affinity seen with these two molecules on heparin–Sepharose is a result of the increased total basic charge of the proteins. However, this increased heparin affinity could still lead to a prolongation of biological half-life due to decreased denaturation and/or proteolysis of the analogues.

Alterations in the second heparin-binding domain revealed that substitution of Arg^{107} with Glu increased the potency of the molecule by 2-fold (Figure 4B and Table I). In contrast, the Ile^{107} derivative was shown to be a weak but full agonist that was almost 100-fold less potent than recombinant bovine bFGF. A synthetic peptide fragment representing residues 106–115 in bFGF has been identified as being important to heparin and receptor binding (Baird et al., 1988). In addition, a recent study has presented evidence that phosphorylation of threonine-112 by the cAMP-dependent protein kinase A increased the receptor binding of bFGF but not its affinity for heparin (Feige & Baird, 1989). Our results with the second domain analogues indicate that this region of the molecule is important and that introduction of a negative charge does increase biological potency. The results of our study indicate that [Glu^{107}]rbFGF has a lowered affinity for heparin as compared to basic FGF and its increased biological activity may be due to a decreased sequestration in the extracellular matrix, leaving more mitogen free for interaction with the high-affinity FGF receptor. In contrast to the Glu^{107} derivative, the Ile^{107} analogue showed a sharp reduction in activity relative to recombinant bovine bFGF without any alteration in the affinity of the molecule for heparin. Such a reduction could be the result of the destabilization of the protein structure by the introduction of the β-branched side chain of isoleucine. Acidic fibroblast growth factor contains an isoleucine at this position, and it is tempting to speculate as to whether this is responsible for some of lowered potency seen for aFGF in the absence of heparin. However, unlike acidic FGF, the Ile^{107} derivative does not demonstrate equal potency to bFGF in the presence of heparin (Figure 5D and Table I, see below).

The third heparin-binding region of FGF has been identified primarily as a result of chemical and enzymatic studies on acidic FGF that indicated the importance of this region to heparin and receptor binding and biological activity (Harper & Lobb, 1988; Lobb, 1988). In addition, sequence analysis of the seven members of the FGF family point to the preponderance of basic residues in this portion of the molecule with the concomitant possibility of interaction with sulfated glycosaminoglycans (Delli Bovi et al., 1987; Esch et al., 1985; Finch et al., 1989; Gimenez-Gallego et al., 1985; Marics et al., 1989; Smith et al., 1988; Taira et al., 1987; Zhan et al., 1988). Replacement of glutamine-123 with glutamic acid resulted in a dramatic alteration in both the ED_{50} and the maximum mitogenic response of the analogue (Figure 4C and Table I). An examination of the sequences of the seven known members of the FGF family reveals a total absence of negatively charged amino acids in this region. Introduction of a negative charge into this very basic region of the protein may be responsible for altering the molecule's interaction with heparin and its receptor with a concomitant change in biological potency. On the other hand, replacement of the neutral amino acids glutamine-123 and leucine-126 with basic residues, a substitution present in several other FGF family members, to generate the $Lys^{123,126}$ analogue only had a modest effect on biological activity (Figure 4C and Table I). Addition of two extra basic residues in this highly basic region did not appear to have a substantial impact on FGF activity. Harper and Lobb have reported that reductive methylation of lysine-118 in acidic FGF (a position that corresponds to residue 125 in basic FGF) resulted in a substantial decrease in both the heparin-binding ability and the biological potency of the mitogen (Harper & Lobb, 1988). In addition, replacement of this lysine residue in acidic FGF with a glutamic acid leads to a derivative with a marked reduction in heparin affinity and biological activity (Burgess et al., 1990). The C-terminal FGF mutants described in this study do not demonstrate the same large reduction in heparin affinity. One possible explanation for this difference is the highly basic nature of bFGF as op-

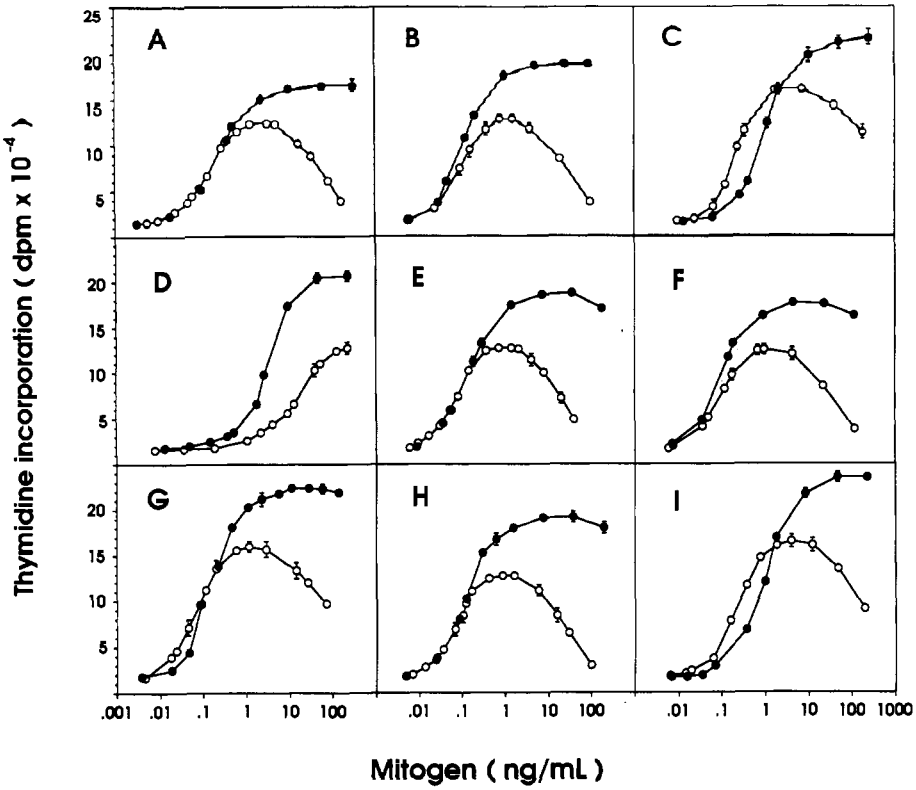

FIGURE 5: Effect of heparin on FGF analogue stimulated mitogenesis in quiescent human foreskin fibroblasts. The figure shows the dose response curves in the presence (●) and absence (○) of 100 μg/mL heparin for recombinant bovine bFGF (panel A), [Asn^{19}]rbFGF (panel B), [Arg^{19}]rbFGF (panel C), [Ile^{107}]rbFGF (panel D), [Glu^{107}]rbFGF (panel E), [$Lys^{123,126}$]rbFGF (panel F), [$Glu^{107,123}$]rbFGF (panel G), [Glu^{123}]rbFGF (panel H), and [Arg^{19},$Lys^{123,126}$]rbFGF (panel I).

posed to aFGF. A recent study involving deletion mutants of basic FGF suggests that it is possible to change biological activity of bFGF without substantially altering heparin affinity and confirms that the carboxyl-terminal region of bFGF is critical for biological activity (Seno et al., 1990).

Multiple heparin-binding domain replacements were constructed to generate the analogues [$Glu^{107,123}$]rbFGF and [Arg^{19},$Lys^{123,126}$]rbFGF in order to study possible additive effects of domain substitutions. The $Glu^{107,123}$ analogue was virtually identical with the Glu^{107} derivative in the mitogenicity assay (Figure 4D and Table I) and, as such, appeared to demonstrate no additive effects of substitutions in heparin-binding domains. In addition, the activity of the Arg^{19},$Lys^{123,126}$ molecule also did not appear to deviate from that seen with the Arg^{19} analogue. The reason for the apparent reduction in activity of the Glu^{123} substitution by combination with Glu^{107} is not known. One possibility is that the introduction of negative charge into two of the three heparin-binding domains may alter the stability or receptor interaction of the molecule.

When the FGF analogues were analyzed for the effect of soluble heparin (100 μg/mL) on their mitogenic activity, there was a dramatic increase in total mitogenic response with little or no effect on the ED_{50} values (Figure 5 and Table I). In addition, the normal bell-shaped biphasic dose response curve was converted to one where the mitogenic response reached a maximum value and then plateaued despite increasing growth factor concentrations. The reasons for this change are not known but may reflect a change in receptor interaction or mitogen stability. Other studies have reported that heparin or heparan sulfate either increased, decreased, or had no effect on the biological activity of basic FGF (Gospodarowicz & Cheng, 1986; Moscatelli, 1987; Saksela et al., 1988; Shipley et al., 1989; Sommer & Rifkin, 1989). In addition, a recent paper has presented evidence that heparin increases the affinity of the human FGF receptors *flg* and *bek* for acidic FGF (Kaplow et al., 1990). The data in this study demonstrate an increase in the biological activity of recombinant basic FGF and the FGF analogues in the presence of soluble heparin without significant alterations in ED_{50} values. The exceptions to the latter part of the preceding statement deal with the Ile^{107}, Arg^{19}, and Arg^{19},$Lys^{123,126}$ derivatives. The presence of soluble heparin increased the potency of the Ile^{107} analogue by 6.5-fold but still leave it as a weak full agonist and may reflect a stabilization of the molecule (Figure 5D and Table I). On the other hand, heparin shifts the dose response curves of the Arg^{19} and Arg^{19},$Lys^{123,126}$ derivatives to the right, possibly reflecting the increased avidity of these two analogues for heparin (Figure 5C,I and Table I) and a decreased availability of mitogen for binding to the high-affinity FGF receptor. Interestingly, the results of the Arg^{19},$Lys^{123,126}$ derivative in the presence of heparin (Figure 5I) appear to reflect an additive effect of the two separate domain changes on the ED_{50} value of the analogue (Figure 5C,F).

A follow-up experiment examining the effect of the dose of heparin on FGF analogue mitogenicity revealed no differences between heparin concentrations ranging from 15 to 1000 μg/mL and that heparin at all doses had only a minor effect on basal thymidine incorporation in human foreskin fibroblasts (data not shown). Additional experiments with various types of heparin established that the heparin effect seen with bFGF and this particular cell type was not due to the type of heparin used (data not shown). In addition, the effect of heparin on the mitogenic effect of other growth factors on fibroblasts was tested. Soluble heparin (100 μg/mL) had no effect on the dose response curve or maximum mitogenic response of platelet-derived growth factor and insulin-like growth factors I and II. Heparin increased the potency of bovine

acidic FGF to that of recombinant bovine bFGF without altering its maximum mitogenic response (Table I). A small increase in mitogenic activity was seen when heparin was used in combination with epidermal growth factor (data not shown).

The nature of structure–function relationships in the fibroblast growth factor family is just beginning to be addressed. The initial results of such studies have allowed researchers to draw some preliminary conclusions. Heparin protects the FGF molecule from proteolytic degradation and also stabilizes the molecule (Damon et al., 1989; Gospodarowicz & Cheng, 1986; Lobb, 1988; Rosengart et al., 1988; Saksela et al., 1988; Sommer & Rifkin, 1989). More important, however, is the role that heparin plays in the bioavailability of FGF in vivo. Binding to heparin-like molecules on endothelial cells protects the FGF from proteases such as plasmin that are generated during processes such as inflammation and angiogenesis (Sommer & Rifkin, 1989). In addition, the matrix-bound FGF appears to serve as a reservoir of mitogen. As such, the ability to bind heparin clearly regulates the distribution, concentration, and half-life of FGF as well as its biological activity. Any alteration in this heparin binding would be expected to affect biological activity. Harper and Lobb have speculated that FGF action in vivo may actually involve a ternary complex between mitogen, heparin, and the receptor (Harper & Lobb, 1988), which is analogous to the binding of antithrombin III and thrombin to heparin via a ternary complex as a prerequisite for the acceleration of the antithrombin–thrombin reaction (Danielsson et al., 1986). Whether the heparin-binding and receptor-binding domains of the FGF molecule lie within the same linear amino acid sequence as is the case for apolipoprotein E (Weisgraber et al., 1986) is not yet clear (Baird et al., 1988; Kurokawa et al., 1989; Schubert et al., 1987).

This study has examined the effects of charge alterations in the three putative heparin-binding domains of basic fibroblast growth factor on its biological activity. These mutations resulted in moderate changes in heparin binding and/or biological activity of the molecules. Alterations in heparin affinity could lead to shifts in the equilibrium between the high- and low-affinity FGF receptors and result in changes in mitogen sequestration in the extracellular matrix, down regulation of the high-affinity FGF receptor, and mitogen stability. Alternatively, the substitutions made in this study may play more of a role in the conformation of the molecule as opposed to being directly involved in heparin and/or receptor interaction. Less likely is the possibility that the analogues are causing a decrease in cell cycle time resulting in more rapid cell division. At the present time, it is not possible to conclude which of these possibilities are responsible for the altered biological activities of the FGF analogues detailed in this study. The findings in this study provide insight into FGF structure–function relationships and will aid in the development of more active and selective fibroblast growth factor molecules.

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Registry No. rbFGF, 133419-61-1; [Asn^{19}]rbFGF, 133419-62-2; [Arg^{19}]rbFGF, 133419-63-3; [Glu^{107}]rbFGF, 133419-64-4; [Ile^{107}]-rbFGF, 133419-67-7; [Glu^{123}]rbFGF, 133419-65-5; [$Lys^{123,126}$]rbFGF, 133419-68-8; [$Glu^{107,123}$]rbFGF, 133419-66-6; [Arg^{19},$Lys^{123,126}$]rbFGF, 133419-69-9; basic FGF, 106096-93-9; recombinant bovine basic FGF, 99400-52-9; bovine acidic FGF, 100215-04-1; recombinant human Met-Arg-Leu-bFGF, 133419-60-0; heparin, 9005-49-6.

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