Site-Directed Mutagenesis and Molecular Modeling Identify a Crucial Amino Acid in Specifying the Heparin Affinity of FGF-1†

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ABSTRACT: Heparin potentiates the mitogenic activity of FGF-1 by increasing the affinity for its receptor and by extending its biological half-life. During the course of labeling human FGF-1 with $Na^{125}I$ and chloramine T, it was observed that the protein lost its ability to bind to heparin. In contrast, bovine FGF-1 retained its heparin affinity even after iodination. To localize the region responsible for the lost heparin affinity, chimeric FGF-1 proteins were constructed from human and bovine FGF-1 expression constructs and tested for their heparin affinity after iodination. The results showed that the C-terminal region of human FGF-1 was responsible for the loss of heparin affinity. This region harbors a single tyrosine residue in human FGF-1 in contrast to a phenylalanine at this position in bovine FGF-1. Mutating this tyrosine residue in the human FGF-1 sequence to phenylalanine did not restore the heparin affinity of the iodinated protein. Likewise, changing the phenylalanine to tyrosine in the bovine FGF-1 did not reduce the ability of the iodinated protein to bind to heparin. In contrast, a mutant human FGF-1 that has cysteine-131 replaced with serine (C131S) was able to bind to heparin even after iodination while bovine FGF-1 (S131C) lost its binding affinity to heparin upon iodination. In addition, the human FGF-1 C131S mutant showed a decrease in homodimer formation when exposed to $CuCl_2$. Molecular modeling showed that the heparin-binding domain of FGF-1 includes cysteine-131 and that cysteine-131, upon oxidation to cysteic acid during the iodination procedures, would interact with lysine-126 and lysine-132. This interaction alters the conformation of the basic residues such that they no longer bind to heparin.

The fibroblast growth factor (FGF)[1] family presently consists of 17 FGF proteins that share 30−70% sequence identity (*1*−*5*). These proteins have considerable overlap in their biological activities toward cells of neuroectodermal and mesodermal origin. The distinct biological effects exerted by these growth factors are mediated by the cell surface high-affinity FGF receptors (FGFR) that constitute a subclass of receptor tyrosine kinases (*6*). In addition to the high-affinity receptors, FGFs also bind to a low-affinity component of cells which has been shown to be heparan sulfate proteoglycans (HSPG) (*7*−*10*). The ability to bind heparin is a feature shared by all FGF family members. The mitogenic activity of FGF-1 has been shown to be potentiated by heparin (*11, 12*). In addition, it has been shown that heparin protects FGF-1 and FGF-2 from heat and acid inactivation and from proteolytic modification (*13*−*16*). The binding of growth factors to proteoglycans is thought to have an important regulatory role as well (*17, 18*). Indeed, the binding of FGFs to HSPGs or heparin has important physiological consequences on their biological activities (*19*−*22*).

Because the interaction between FGF and heparin strongly influences the growth factors' biological activities, the three-dimensional structure of FGFs is important in our understanding of the nature of this interaction at the molecular level. A number of X-ray crystal structures of FGF-1 and FGF-2 have been obtained (*23*−*29*). The FGFs exhibit an overall structure comprising 12 antiparallel β-strands linked to form a β-barrel with an approximate 3-fold internal symmetry. A striking feature from the structures of FGF-2 is a cluster of basic residues on the face of the protein that is postulated to be a site of heparin interaction (*23*−*26*). The basic nature of these residues is conserved in this region of FGF-1 at both the primary and secondary structure levels as well. This site was shown to have a bound sulfate ion in the three-dimensional structures of both FGF-1 and FGF-2, which suggests that this is the binding site for the sulfate group of heparin (*27*−*29*). Chemical modification and site-directed mutagenesis studies conducted on FGF-1 have demonstrated the importance of lysine-132 in heparin binding and biological function (*30*−*32*). These results are consistent with the X-ray crystal structure of FGF-1 (*27*−*29*). However, the actual heparin-binding site(s) of FGF-1 has not been systematically analyzed by experimental approaches.

The FGF family displays a conservation of two cysteine residues, cysteine-30 and cysteine-97, in their primary amino acid sequences. In addition to the two conserved cysteine residues, human FGF-1 contains an additional cysteine at

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[1] Abbreviations: FGF, fibroblast growth factor; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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position 131. The function of these cysteine residues and the selective evolutionary pressure for their conservation are not clear. However, a number of studies using human FGF-1 have provided some useful insights into this dilemma. Site-directed mutagenesis of any one of the three cysteine residues in FGF-1 to serine results in proteins that remain highly active. Thus none of the three possible intramolecular disulfide bonds are required for mitogenic activity (*33*). In addition, formation of a disulfide bond between the two conserved cysteine residues results in a virtually inactive product that is subsequently reactivated by reduction. Therefore, cysteine residues do not form intramolecular disulfide bonds in the active conformation of FGF-1. Furthermore, mutants of FGF-1 in which any two or all three cysteine residues are substituted by serines are more active, have longer activity half-lives, and are less heparin dependent than wild-type FGF-1 (*34*). In contrast, wild-type FGF-1 and the three mutants that each retains two cysteine residues are inactivated more rapidly in the absence of heparin via a nonproteolytic mechanism but are stabilized by heparin. The copper-catalyzed oxidation of FGF-1 results in the formation of inactive FGF-1 homodimers that can be reduced with dithiothreitol to give biologically active monomers (*35*). These results suggest that the inactivation of wild-type FGF-1 is due to its oxidation which results in the formation of inactive homodimers. It has been demonstrated that FGF-1 is released from cells grown under stressed conditions (*36, 37*). However, the secreted FGF-1 has a decreased affinity for heparin and appears as an inactive homodimer that has to be treated with reducing agents for conversion to biologically active monomers. Additionally, the cysteine residues of FGF-1, particularly cysteine-30, are necessary for its release in response to heat shock (*38, 39*). It is thus believed that cysteine residues do play a significant role in FGF-1 biology through their participation in the nonconventional release of the growth factor from cells and regulation of its activity once outside.

Although previous X-ray studies and chemical and genetic modifications of FGF-1 allowed the implication of amino acids 126−132 as the putative heparin-binding site, the fortuitous findings of the difference in heparin affinity between iodinated human and bovine FGF-1 allow us to perform thorough mix-and-match studies to identify the heparin-binding region(s). Additional site-directed mutagenesis of residues different among human, bovine, and urodele amphibian FGF-1 proteins further identify cysteine-131 as a crucial amino acid in the heparin-binding domain.

MATERIALS AND METHODS

Construction of Wild-Type, Mutant, and Chimeric FGF-1 Expression Vectors. The coding regions of human and bovine FGF-1 were amplified from existing cDNA clones by the polymerase chain reaction (PCR) using oligonucleotide primers that had restriction enzyme sites incorporated into them to facilitate cloning into the prokaryotic expression vector pET20b(+) (Novagen). The cloning, expression, and purification of newt FGF-1 is described elsewhere (*40*). The human FGF-1 cDNA (*41*) was amplified with the sense primer BEF1 (5′-TGA AGC CAT ATG GCT GAA GGG GA-3′) and the antisense primer HBGF605 (5′-AGA TCT CTT TAA TCA GAA GAG ACT-3′). The bovine FGF-1 cDNA (*42*) was amplified with the sense primer BEF3 (5′-TGC TGA CAT ATG GCT GAA GGA GA-3′) and the antisense primer BER3 (5′-CAA CAG ATC TCT TTA ATC AGA GGA GAC-3′). PCR reactions were carried out in 10 mM Tris-HCl, pH 8.5, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% Tween 20, 0.01% Triton X-100, 0.25 mM dNTPs, 10 pmol of each primer, and 2 units of *Taq* polymerase. The reaction was initially denatured at 95 °C for 2 min followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 42 °C for 1 min (50 °C for human FGF-1), extension at 72 °C for 1 min, and finishing with a final extension at 72 °C for 7 min. The resulting 489 bp amplicon from the bovine FGF-1 cDNA template was gel purified, digested with *Nde*I and *Bgl*II, and cloned into pET20b(+), which was previously digested with *Nde*I and *Bam*HI, to yield the bovine FGF-1 expression vector, pETbovFGF-1. The gel-purified 485 bp amplicon from the human FGF-1 cDNA template was first cloned into pBluescript previously digested with *Sma*I. The resultant plasmid was then digested with *Nde*I and *Bgl*II and the human FGF-1 cDNA insert cloned into pET20b(+) to yield the human FGF-1 expression vector pEThumFGF-1.

The amino acid at position 139 in the human FGF-1 sequence was mutated from a tyrosine to a phenylalanine (human Y139F), and the homologous amino acid in bovine and newt FGF-1 sequences was mutated from a phenylalanine to a tyrosine (bovine F139Y and newt F139Y) using the Transformer Site-Directed Mutagenesis Kit following the manufacturer's protocol (Clontech). The mutagenesis procedure (*43*) was performed directly on the human, bovine, and newt FGF-1 expression vectors described above. The antisense selection primer used for the plasmid DNAs was 5′-GCA GCC ACT AGT AAC AGG ATT-3′, which changes an *Alwn*I restriction site within pET20b(+) to an *Spe*I site. The antisense mutagenic primers used for the human, bovine, and newt FGF-1 sequences were 5′-CTT TCT GGC CAA AGT GAG TCC-3′, 5′-CTT TCT GGC CGT AGT GAG TCC-3′, and 5′-CTT TTT GGC CAT AGT GGG TCC-3′, respectively. The selection primer was also used in mutagenesis reactions for changing cysteine-131 of human FGF-1 to serine (human C131S) and serine-131 of bovine FGF-1 to cysteine (bovine S131C). The mutagenic primers used for the human C131S and bovine S131C sequences were 5′-GGA CCG CGT TTG GAG CTC CCA TTC-3′ and 5′-GGA CCG AGT TTA CAC CTT CCG TTC-3′, respectively. The mutated plasmids were verified by restriction enzyme analysis and direct sequencing of plasmid DNA.

Chimeric FGF-1 proteins were constructed directly from the human and bovine FGF-1 expression vectors described above (also see Figure 4). pEThumFGF-1 and pETbovFGF-1 were digested with *Bgl*II and *Nco*I in combination or with *Pvu*II alone and electrophoresed on a 1% agarose gel (*Bgl*II and *Nco*I have unique sites in the expression vectors yet are common in both; *Pvu*II sites are located once in the pET20b-(+) vector sequence and once in the cDNA insert sequence of both expression vectors). The 3759 bp *Bgl*II−*Nco*I fragment from pEThumFGF-1 was ligated to the 341 bp *Bgl*II−*Nco*I fragment from pETbovFGF-1 to make the chimera BBH. The reciprocal ligation (the 3759 bp fragment from pETbovFGF-1 ligated to the 341 bp fragment from pEThumFGF-1) resulted in the chimera HHB. Chimera BHH was constructed by ligating the 3186 bp *Pvu*II fragment from pEThumFGF-1 to the 914 bp *Pvu*II fragment from pETbovFGF-1, and HBB was constructed by ligating the 3186

bp *Pvu*II fragment from pETbovFGF-1 to the 914 bp *Pvu*II fragment from pEThumFGF-1. Standard cloning techniques were used in the construction of chimeric DNA molecules (*44*) and were verified by restriction enzyme analysis and direct sequencing of plasmid DNA.

Purification and Identification of FGF-1 Proteins. The FGF-1 expression vectors were used to transform the bacterial expression host BL21(DE3)pLysS and then induced and purified as previously described (*40*). In addition, 15 μL samples of the elution fractions from the heparin−Sepharose column were analyzed by 15% SDS−PAGE and silver staining to identify the protein-containing fractions and their purity. The first 29 amino acid residues of newt wild-type FGF-1 were determined using Protein Sequencer 475A (PE Applied Biosystems, Foster City, CA). The determined N-terminal amino acid sequence is identical to the predicted sequence saved the initiating methionine, which was cleaved following protein synthesis. We have also used matrix-assisted laser desorption ionization (MALDI) mass spectrometry to determine the molecular weights of the purified recombinant proteins. The LASERMAT 2000 Mass Analyzer (Finnigan MAT, San Jose, CA) was used for such measurements. The predicted molecular weight, observed molecular weight, and the percentage error are listed for human wild-type FGF-1 (17 330.0; 17 331.3; +0.008%), human mutant FGF-1 C131S (17 314.0; 17 311.2; −0.016%), and newt mutant FGF-1 F139Y (17 414.8; 17 410.3; −0.026%). Both protein sequencing analysis and molecular weight determination by mass spectrometry help to authenticate the identity of each recombinant protein. Bovine native FGF-1 (R&D Systems, Minneapolis, MN) represents a 140 amino acid form of bovine FGF-1 (amino acids 15−154).

Iodination of FGF-1 Proteins. The iodination procedure performed on all FGF-1 proteins is described elsewhere (*40*). Briefly, 2 μg of FGF-1 protein was iodinated with $Na^{125}I$ using chloramine T. The specific activities of iodinated FGF-1 proteins ranged from (1 to 2) $\times$ 10^8 cpm/μg. The iodination reactions were placed on a heparin−agarose column (0.2 mL of a 50% slurry of heparin−agarose in a pipet tip packed with siliconized glass wool) which was equilibrated with wash buffer [20 mM HEPES, pH 7.3, 0.4 M NaCl, 0.2% bovine serum albumin (BSA)] and 0.5 mL wash fractions collected. Bound FGF-1 was eluted off the column with elution buffer (20 mM HEPES, pH 7.3, 2.0 M NaCl, 0.2% BSA) as 0.1 mL fractions. The heparin affinity chromatography was carried out at 4 °C. Under normal conditions using gradient salt elution, wild-type human, bovine, and newt FGF-1 proteins would come off the column at 1.0 M NaCl concentration. Aliquots representing 1% of each fraction were subjected to 15% SDS−PAGE to obtain a wash and elution fraction profile. The gel was then dried and exposed to X-ray film. Bands representing iodinated FGF-1 on the resulting autoradiographs were quantitated on an LKB laser densitometer. The percent of iodinated FGF-1 in each fraction was calculated by dividing the value of the band in an individual lane on the autoradiograph by the sum of the value of all lanes.

Western Blot Analysis. Human wild-type FGF-1 was used in a mock iodination reaction in which $Na^{125}I$ and chloramine T were not present. The reaction was placed on a heparin−agarose column with wash and elution fractions collected as above. Aliquots representing 5% of the total volume of each fraction were resolved by 15% SDS−PAGE. The proteins were electrophoretically transferred to a nitrocellulose membrane (Schleicher & Schuell) and incubated in blocking buffer [Tris-buffered saline, 0.1% Tween 20 (TTBS) plus 3% BSA] for 1 h at room temperature. The membrane was then incubated with a rabbit anti-FGF-1 polyclonal antibody (Sigma) at a 1:1000 dilution in blocking buffer for 1 h at room temperature. After extensive washing in TTBS, the membrane was incubated with a horseradish peroxidase conjugated anti-rabbit IgG antibody (Amersham) at a dilution of 1:1000 in blocking buffer for 1 h at room temperature. The membrane was again extensively washed in TTBS followed by detection of the proteins by ECL (Amersham) and exposure to an X-ray film.

Mitogen Assays. Swiss/3T3 cells were maintained in Dulbecco's-modified Eagle's medium (DMEM) supplemented with 10% calf serum and penicillin/streptomycin. Cells were seeded in 24-well plates at a density of 2×10^4 cells/well. At ~80% confluency, the cells were washed once with phosphate-buffered saline (PBS) and placed in low-serum media (DMEM, 0.5% calf serum, penicillin/streptomycin) for 24 h. The cells were stimulated with recombinant human or recombinant bovine FGF-1 in the presence of heparin (10 μg/mL) for 20 h and then pulsed with 1 μCi of [^{3}H]thymidine (NEN) for 6 h. As a positive control, calf serum at a final concentration of 10% was used. The labeling was terminated by washing the cells twice with 1 mL of PBS, twice with 1 mL of cold (4 °C) 5% trichloroacetic acid, and twice again with 1 mL of PBS. Cells were then solubilized in 0.8 mL of 0.25 M NaOH, and a 0.1 mL aliquot was counted in a Beckman scintillation counter.

$CuCl_2$ Treatment of Human and Bovine FGF-1 Wild-Type and Mutant Proteins. Two micrograms of FGF-1 was incubated with Tris-HCl, pH 7.4, at a final concentration of 20 mM and $CuCl_2$ at a final concentration of 10 mM in a total volume of 20 μL for 30 min at room temperature. The reaction was stopped by the addition of nonreducing 2× SDS sample buffer containing 10 mM EDTA and directly loaded on a 15% SDS−PAGE gel without boiling. The resulting gel was then subjected to Western blotting as above to visualize the proteins. For some experiments where protein samples were treated with $CuCl_2$, dithiothreitol was also added at a final concentration of 100 mM and the samples were boiled prior to SDS−PAGE and Western blotting.

Molecular Modeling. The coordinates of the X-ray structure of the FGF−heparin complex (*29*) were used as the initial structure for molecular modeling. This complex is a dimer linked by heparin. The heparin molecule is a decasaccharide, but five of them were disordered; hence only six of them were solved in the X-ray structure. We further energy-minimized both the complexed and uncomplexed FGF molecules to remove unfavorable nonbonded interactions. For the oxidized FGF molecule, we modified the original cysteine-131 to a negatively charged cysteic acid and then rotated manually the side-chain dihedrals of the basic residues that were within 5 Å of cysteic acid, such that they would come in close proximity to it. Finally, the whole protein was subjected to energy minimization to relax the protein conformation. All calculations were done on a Silicon Graphics Indigo II workstation using the molecular modeling package InsightII from Molecular Simulation, Inc.

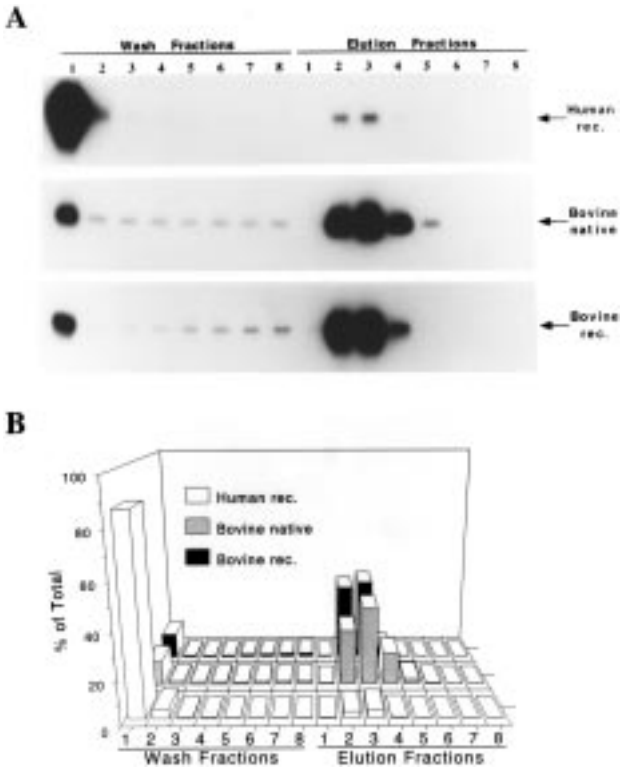

FIGURE 1: Iodinated human FGF-1 lost the majority of its heparin affinity. Human recombinant, bovine native, and bovine recombinant FGF-1 were subjected to iodination reactions using $Na^{125}I$ and chloramine T, and the labeled proteins were separated from unincorporated ^{125}I by passing the reactions over a heparin−agarose column. (A) Aliquots of the wash and elution fractions from the heparin−agarose columns were subjected to 15% SDS−PAGE, and the resulting gel was dried and exposed to an X-ray film. (B) Bands of ^{125}I-FGF-1 on the autoradiographs shown in (A) were quantitated on a densitometer, and the percent of labeled FGF-1 in each fraction was calculated by dividing the value of a fraction by the sum of the values of all fractions.

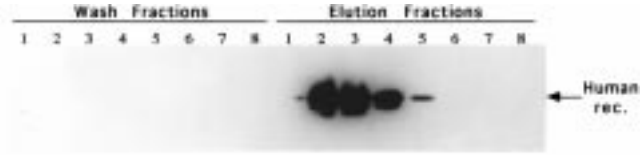

FIGURE 2: Western analysis of heparin−agarose column fractions from a "mock" iodination reaction with human recombinant FGF-1. Human recombinant FGF-1 was subjected to a mock iodination reaction in which chloramine T and $Na^{125}I$ were omitted and the reaction placed on a heparin−agarose column. Aliquots of the wash and elution fractions were subjected to 15% SDS−PAGE, transferred to a nitrocellulose membrane, and examined for the presence of FGF-1 with a polyclonal anti-FGF-1 antibody. Proteins on the membrane were visualized by ECL.

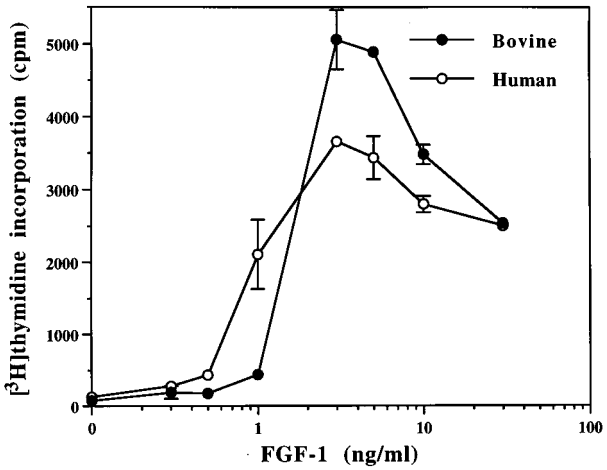

FIGURE 3: Mitogenic response of Swiss/3T3 fibroblast cells to bovine and human FGF-1. Swiss/3T3 cells were grown to ~80% confluency in 24-well plates and then serum starved for 24 h. Bovine (closed circle) or human (open circle) recombinant FGF-1 at the indicated concentration was added directly to the starvation media, and the cells were incubated for 20 h after which time they were pulsed with 1 μCi of [^{3}H]thymidine for 6 h and the amount of radioactivity was determined. Values are the mean cpms of duplicate samples (±SE).

RESULTS

Loss of Heparin Affinity of Recombinant Human FGF-1 upon Iodination. In the course of iodinating human and bovine FGF-1 proteins for use in radioligand binding assays, the labeled protein is separated from unincorporated ^{125}I by placing the reaction on a heparin column. The ability of FGF-1 to bind to heparin has been exploited to purify this protein from recombinant and natural sources. Therefore, passing an iodination reaction involving FGF-1 over a heparin column is a quick and efficient method of eliminating unincorporated ^{125}I. In addition, because FGF-1 is dependent on its binding to heparin for its full biological activity, the ^{125}I-FGF-1 bound and eluted from a heparin column is certain to have retained its biological potential. The free ^{125}I is washed away with a low-salt buffer, and the labeled protein that is adsorbed to the column is eluted off with a high-salt buffer. It was consistently observed, however, that human recombinant ^{125}I-FGF-1 would lose most (>90%) of its inherent affinity for heparin whereas the bovine native ^{125}I-FGF-1 retained most (>85%) of this binding propensity (Figure 1). This phenomenon was not due to a difference in the size (154 amino acids vs 140 amino acids) or the source (recombinant vs native) of these two proteins as full-length (154 amino acids) recombinant bovine FGF-1 behaved in a manner similar to that of the native bovine FGF-1 when it was iodinated and purified over a heparin−agarose column in that greater than 85% of the labeled protein could be bound to the column and recovered in the elution fractions (Figure 1).

The ability of unlabeled human recombinant FGF-1 to bind to a heparin−agarose column was examined to verify the heparin affinity of this recombinant protein. Human recombinant FGF-1 was subjected to a "mock" iodination reaction in which $Na^{125}I$ and chloramine T were omitted. The mock reaction was placed over a heparin−agarose column, and samples from the wash and elution fractions were subjected to 15% SDS−PAGE, transferred to a nitrocellulose membrane, and reacted with an anti-FGF-1 polyclonal antibody. The resulting Western blot reveals that the unlabeled human recombinant FGF-1 is able to bind to the heparin column as all the protein can be seen coming off in the elution fractions (Figure 2). In addition, this human recombinant FGF-1 is fully capable of eliciting a mitogen response in Swiss/3T3 mouse fibroblasts comparable to that of the bovine recombinant FGF-1 used in these studies (Figure 3).

Chimeric Human/Bovine FGF-1 Proteins. Human and bovine FGF-1 proteins share a high degree of amino acid identity with only 12 different amino acids (Figure 4). To localize the region(s) of human FGF-1 responsible for the

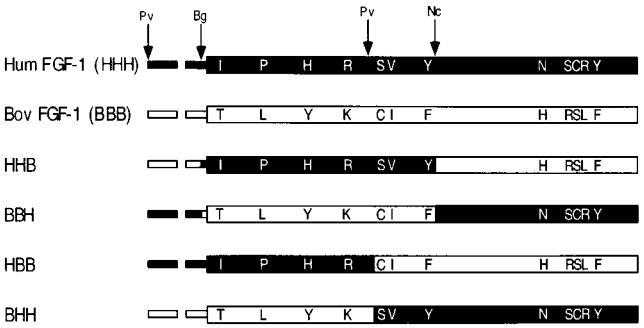

FIGURE 4: Diagram of FGF-1 chimeric proteins illustrating the portions of human and bovine FGF-1 that make up each chimera. The thick lines represent the FGF-1 cDNA insert of the expression vectors, and the thin lines represent vector sequences within the same expression vectors. The letters within the thick lines represent the single-letter abbreviations of the amino acids that differ between human FGF-1 (filled box) and bovine FGF-1 (open box). The restriction enzymes utilized in the construction of the chimeric expression vectors are indicated above the human FGF-1 (HHH) box with their relative positions indicated by the arrows. Abbreviations: Pv, *Pvu*II; Bg, *Bgl*II, Nc, *Nco*I. The space in the small box flanked by the *Pvu*II and *Bgl*II sites represents a gap in the vector sequence of the expression vectors that is omitted in order to show the *Pvu*II site in the vector.

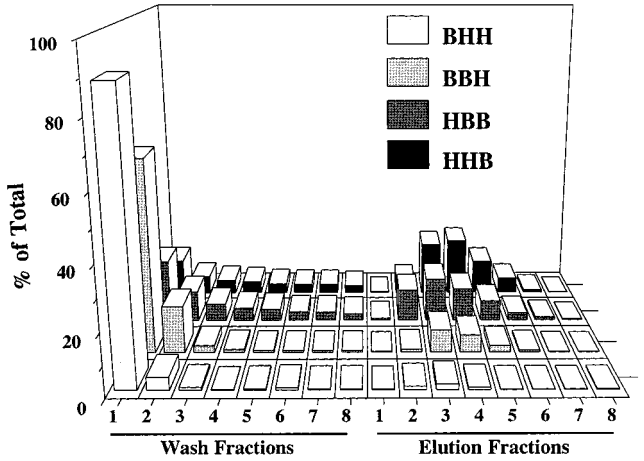

FIGURE 5: Graphical representation of the percent of ^{125}I-FGF present in wash and elution fractions from heparin−agarose columns used to purify the labeled chimeric proteins. BHH, BBH, HBB, and HHB represent the portions of the human (H) and bovine (B) contribution of each chimeric FGF-1 protein.

observed loss of heparin affinity after iodination, a series of chimeric FGF-1 molecules were constructed between human and bovine FGF-1. The chimeric proteins (diagrammed in Figure 4) were purified from *Escherichia coli* and labeled with ^{125}I. The chimeras BHH and BBH exhibit heparin-binding behaviors similar to those of wild-type human FGF-1, while the chimeras HBB and HHB are similar to wild-type bovine FGF-1 (Figure 5). These results identify the carboxyl-terminal portion of FGF-1 as the region responsible for the loss of heparin affinity in the wild-type iodinated human FGF-1. This observation is consistent with previous studies, indicating that this region has a role in heparin binding (*30*−*32*). The secondary structure of FGF-1 supports these claims as this region harbors a number of closely associated basic amino acid side chains (*28*, *29*). This positively charged face of the protein could potentially interact with the negatively charged sulfated groups on heparin.

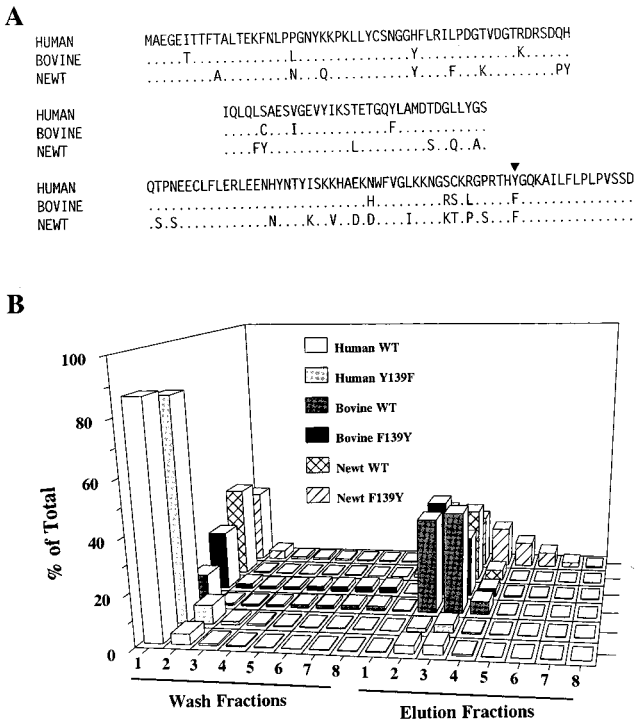

FIGURE 6: Site-directed mutagenesis of residue 139 of human, bovine, and newt FGF-1 and heparin affinity of the iodinated proteins. (A) Amino acid alignment of human (*41*), bovine (*42*), and newt (*40*) recombinant FGF-1. The amino acid at position 139 that was targeted for mutation is indicated by a black triangle. The tyrosine at this position in wild-type (WT) human FGF-1 was changed to a phenylalanine (Y139F), and the phenylalanine at this position in wild-type bovine and newt FGF-1 was changed to tyrosine (F139Y). (B) Graphical representation of the percent of ^{125}I-FGF-1 present in wash and elution fractions from heparin−agarose columns used to purify the indicated labeled proteins.

Site-Directed Mutagenesis of Residue 139 in FGF-1 Proteins. The putative heparin-binding domain within human FGF-1 possesses a tyrosine at position 139. The amino acid at this position in the bovine sequence is a phenylalanine and would not be a substrate for iodination (Figure 6A). We speculated that iodinated tyrosine-139 of human FGF-1 may interfere with the labeled protein's ability to interact with heparin. To test this hypothesis, tyrosine-139 in human FGF-1 was mutated to a phenylalanine (Y139F), and a reciprocal phenylalanine-to-tyrosine change was introduced at the corresponding position in bovine FGF-1 (F139Y). However, the amino acid replacement engineered at residue 139 did not change the heparin affinity profile of the iodinated mutant proteins, as they are indistinguishable from their wild-type counterparts in this respect (Figure 6B). It should be pointed out that there is an additional tyrosine in the human FGF-1 sequence at position 78 which in the bovine sequence is a phenylalanine. However, wild-type newt FGF-1 also harbors a tyrosine residue at this position yet exhibits a heparin-binding profile similar to that of bovine FGF-1 (Figure 6B). Hence, tyrosine-78 in the human FGF-1 sequence is not expected to contribute to the loss of heparin affinity of iodinated human FGF-1. In addition, changing the phenylalanine at position 139 in the newt sequence to tyrosine did not reduce the ability of the labeled protein to bind to heparin (Figure 6B). Therefore, substitution of a hydrogen atom with a bulkier iodine atom in either tyrosine-78 or tyrosine-139 is not the cause for iodinated human FGF-1 to lose its heparin affinity.

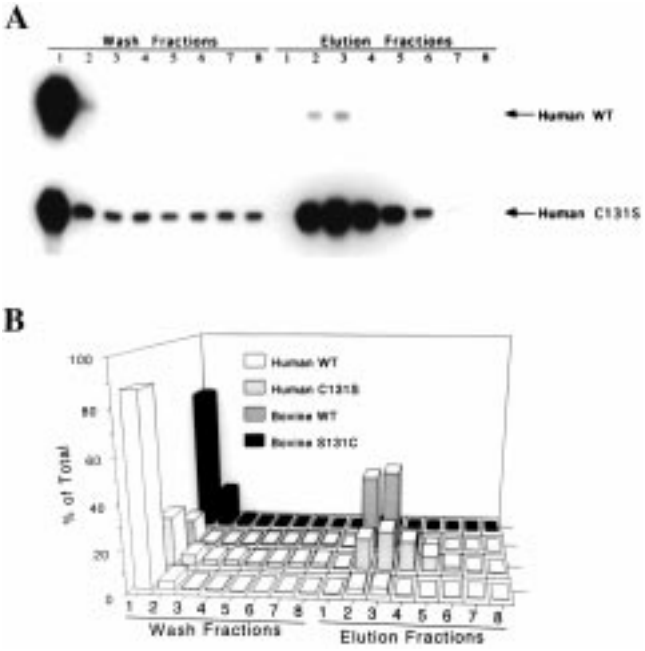

FIGURE 7: Heparin affinities of iodinated human C131S and bovine S131C FGF-1 mutant proteins. Human and bovine recombinant FGF-1 mutant proteins were subjected to iodination reactions using chloramine T, and the labeled proteins were separated from unincorporated ^{125}I by passing the reactions over a heparin−agarose column. (A) Aliquots of the wash and elution fractions from the heparin−agarose columns were subjected to 15% SDS−PAGE, and the resulting gel was dried and exposed to an X-ray film. (B) Bands of ^{125}I-FGF-1 on the autoradiographs shown in (A) were quantitated on a densitometer, and the percent of labeled FGF-1 in each fraction was calculated by dividing the value of a fraction by the sum of the values of all fractions.

Cysteine-131 of Human FGF-1 Plays a Role in the Regulation of Its Heparin Affinity. A cysteine residue is located in the putative heparin-binding domain of human FGF-1 whereas a serine residue resides at this position in bovine FGF-1. We reasoned that this cysteine residue in the human FGF-1 might be involved in the loss of heparin affinity in the iodinated protein. Thus, cysteine-131 of human FGF-1 was changed to a serine residue (human C131S) and serine-131 of bovine FGF-1 was changed to a cysteine residue (bovine S131C). When both mutant proteins are iodinated and passed over a heparin−agarose column, a significant amount of human C131S was recovered in the elution fractions in comparison to wild-type human FGF-1 (Figure 7A). In contrast, iodinated bovine S131C showed a significant decrease in the amount recovered in the elution fractions that is reminiscent of the profile observed for wild-type human FGF-1 (Figure 7B). This suggests that a cysteine residue at position 131 is solely responsible for the loss of heparin affinity in labeled FGF-1 proteins.

The harsh oxidative conditions that the FGF-1 proteins are subjected to during an iodination reaction may cause them to form homodimers with reduced heparin affinity. The presence of homodimers in the wash fractions of human C131S and bovine S131C was tested by analyzing aliquots of the appropriate wash fractions on a nonreducing SDS gel. Figure 8 shows that, under nonreducing conditions, labeled proteins from heparin−agarose wash fractions migrate with sizes expected of FGF-1 monomers. No dimer formation was detected under this oxidative condition. As a control, labeled

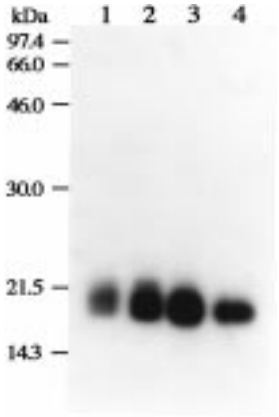

FIGURE 8: Nonreducing SDS−PAGE analysis of iodinated human C131S and bovine S131C mutant FGF-1 proteins. Equal cpms of wash fraction 1 of human C131S (lane 1) and bovine S131C (lane 3), elution fraction 3 of human C131S (lane 2), and elution fraction 2 of bovine S131C (lane 4) of heparin−agarose columns used to purify the iodinated proteins were analyzed by 15% nonreducing SDS−PAGE. The gel was dried and exposed to an X-ray film. The heparin−agarose column fractions examined are from the same iodination reactions shown in Figure 7. Molecular mass markers are in kDa.

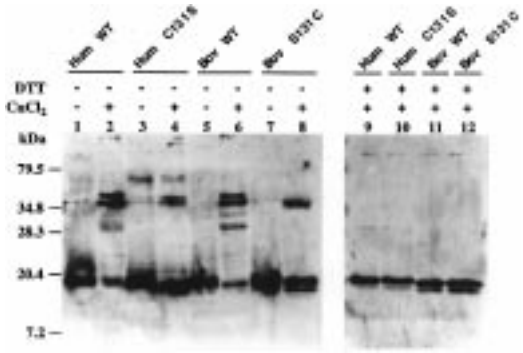

FIGURE 9: $CuCl_2$ treatment of human and bovine wild-type and mutant FGF-1 proteins. Human wild-type (lanes 1, 2, and 9), human C131S mutant (lanes 3, 4, and 10), bovine wild type (lanes 5, 6, and 11), and bovine S131C mutant (lanes 7, 8, and 12) were incubated in the absence (lanes 1, 3, 5, and 7) or presence (lanes 2, 4, 6, and 8−12) of $CuCl_2$. The reactions were either without (lanes 1−8) or with (lanes 9−12) the addition of dithiothreitol (DTT). The reaction products were separated by 15% nonreducing SDS−PAGE. Proteins were transferred to a nitrocellulose membrane, reacted with an anti-FGF-1 antibody, and visualized with ECL. Molecular mass markers are indicated in kDa.

proteins from the elution fractions are also observed to run as FGF-1 monomers.

Finally, the capacity of wild-type human and bovine FGF-1 proteins as well as the human C131S and bovine S131C mutant FGF-1 proteins to form dimers was tested by treating them with $CuCl_2$, which has been previously shown to induce dimer formation of human FGF-1 (*35*). As expected, wild-type human FGF-1 migrated with a size expected of monomers in the absence of $CuCl_2$, but in its presence there was a dramatic shift in the vast majority to a size corresponding to the size of dimers (Figure 9, lanes 1 and 2). Interestingly, many fewer dimers were observed when the human C131S mutant was treated with $CuCl_2$ (lanes 3 and 4), suggesting that cysteine-131 is responsible for the formation of most dimers induced by $CuCl_2$ treatment. This, in turn, suggests that the two conservative cysteine residues, cysteine-30 and cysteine-97, do not contribute significantly

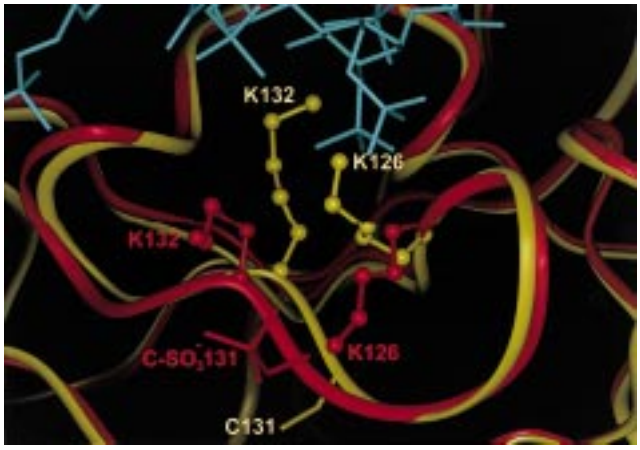

FIGURE 10: Comparison of the molecular structures of the native and the oxidized FGF-1 proteins. The native and the oxidized FGF-1 molecules are shown in yellow and red, respectively. Heparin is shown in cyan. The backbones of FGF-1 are rendered in a ribbon model, while the basic residues lysine-126 (K126) and lysine-132 (K132) are shown in a ball-and-stick model. C-SO_3^- denotes cysteic acid and reflects its negative charge. In the oxidized form, the negatively charged cysteic acid interacts with both K126 and K132 through electrostatic attraction and forces both basic residues to adopt new orientations that are unfavorable for heparin binding.

in the dimer formation. Consistent with this observation, both the wild-type and S131C bovine FGF-1 proteins, which have an additional cysteine at position 61, form dimers in the presence of $CuCl_2$ to a degree that is similar to that of wild-type human FGF-1 (lanes 5−8). Subsequent reduction of the $CuCl_2$-treated FGF-1 proteins with dithiothreitol showed that the dimer formation is a reversible reaction (lanes 9−12).

Oxidation of Cysteine-131 to Cysteic Acid Results in the Loss of Heparin Affinity. To further explore the mechanism of the loss of heparin affinity of human FGF-1 upon iodination, we looked into the molecular structures of both native and iodinated FGF-1 proteins by computer modeling. As shown in Figure 10, the major differences between the oxidized and the native FGF-1 are the change of the orientations of the side chains of lysine-126 and lysine-132 that have been shown to be important in heparin binding. In the native FGF−heparin complex, the neutral cysteine-131 buries inside the protein and has no direct interaction with any of the basic residues that are involved in heparin binding. Under the oxidative conditions during iodination, cysteine-131 is oxidized to a negatively charged cysteic acid. Concomitantly, its side chain rotates out of the pocket to interact electrostatically with the positively charged lysine-126 and lysine-132, forcing their side chains to adopt new orientations unfavorable for heparin binding (Figure 10). It is also interesting to note that our modeling shows that the conformation of the oxidized FGF-1 is essentially identical to that of the native form, as the root-mean-square deviation of Cα atoms between them is around 0.6 Å.

DISCUSSION

The affinity of FGF-1 for heparin has been used to purify the protein from natural as well as recombinant sources (*45*, *46*). It is therefore a convenient method to purify iodinated FGF away from unincorporated ^{125}I. Figure 1 shows that recombinant human FGF-1 is present almost exclusively in the first two wash fractions from a heparin−agarose column whereas both native and recombinant bovine FGF-1 are predominantly seen in the elution fractions. This difference is not due to the source (recombinant vs native) or size (154 amino acids vs 140 amino acids) of the proteins involved. It can be argued that the heparin−agarose column is overloaded with the labeled protein. This does not appear to be the case as the same profile is observed when the first wash fraction of an iodination involving human FGF-1 is passed over a fresh heparin−agarose column (data not shown). The biological activity of the proteins in the elution fractions (both human and bovine) is preserved as both are able to elicit a mitogenic response in Swiss/3T3 fibroblasts (data not shown) and bind to cell surface receptors (*47*) with affinities comparable to those from previously published data. In contrast, the labeled human FGF-1 in the wash fractions is not mitogenically active (data not shown), which is in agreement with the fact that heparin binding is required for FGF-1 mitogenic activity (*11*, *12*).

With the use of human/bovine chimeric FGF-1 proteins, a region corresponding to amino acids 81−154 of human FGF-1 was narrowed down as the region responsible for the decrease in heparin affinity of iodinated human FGF-1. There are five amino acid differences between human and bovine FGF-1 in this region. The tyrosine-139 in human FGF-1 is a potential substrate for iodination and is within a region of basic amino acids that are believed to play a role in heparin binding (*28*, *29*). In addition, all the tyrosines except the one at position 111 have been calculated to be solvent exposed in FGF-1 (48); thus tyrosine-139 would be accessible to modification with ^{125}I. However, a mutant human FGF-1 with a phenylalanine at position 139 (Y139F) did not recover its heparin affinity after an iodination reaction. Likewise, a bovine FGF-1 mutant with the reciprocal amino acid change at this position (F139Y) was indistinguishable from wild-type bovine FGF-1 when iodinated and passed over a heparin−agarose column. Therefore, it is concluded that iodination of tyrosine-139 of FGF-1 is not the cause for interference with its heparin affinity.

Another candidate difference between the human and bovine FGF-1 sequences within this region is cysteine-131 in human, which is a serine in bovine FGF-1. Since an oxidation reaction is involved in the labeling of proteins with ^{125}I, it was hypothesized that the loss of heparin affinity by iodinated wild-type human FGF-1 may be attributed to the presence of a cysteine residue at position 131. Indeed, iodinated human FGF-1 mutant C131S displays a dramatic increase (>20-fold) in its heparin affinity in contrast to the iodinated wild-type human FGF-1. Moreover, the bovine FGF-1 mutant S131C lost its affinity to heparin after it was iodinated. Thus, the presence of a cysteine residue at position 131 in FGF-1 dramatically affects the ability of the iodinated protein to bind to heparin. However, the mechanism of this cysteine-131-dependent abrogation of heparin affinity does not appear to involve the formation of homodimers as bovine S131C from heparin−agarose wash fractions migrates exclusively as monomers on SDS−PAGE. This result is not entirely unexpected, as the molar amount of dithiothreitol used to quench the iodination reaction is in excess of 150- and 17 500-fold over those of chloramine T and FGF-1, respectively. Therefore, it would be anticipated that any dimers of FGF-1 that are potentially formed during the iodination reaction would be reduced to monomers following the addition of dithiothreitol.

The ability of wild-type human FGF-1 to form dimers in the presence of $CuCl_2$ has been shown previously (*36*). We confirm this finding by showing that the wild-type human FGF-1 used in this study is also capable of forming dimers when treated with $CuCl_2$. In contrast, the human C131S mutant formed dimers to a much lesser extent than its wild-type counterpart, indicating that cysteine-131 participates in the majority of dimers formed by copper-catalyzed oxidation of FGF-1. Wild-type bovine FGF-1, which has an additional cysteine residue in position 61, also formed homodimers after $CuCl_2$ treatment in a manner comparable to that of the wild-type human FGF-1. Surprisingly, the bovine S131C mutant exhibited a reduced capacity to form homodimers similar to the human C131S mutant FGF-1. Both wild-type human and bovine FGF-1 proteins have three cysteine residues in their primary sequence which upon exposure to oxidative reagents (e.g., Cu^{2+}, chloramine T) could lead to the formation of one intramolecular disulfide bridge leaving the other cysteine residue free to form a disulfide bond with another FGF-1 molecule. Since the mutant FGF-1 proteins have an even number of cystine residues, the formation of intramolecular cysteines would not leave an available cysteine residue to form intermolecular disulfide bonds, and hence a decrease in the appearance of oxidation-induced homodimers would be observed.

The ability of both wild-type proteins to readily form homodimers may have some biological significance. When placed under stressed conditions, cells overexpressing human FGF-1 release the protein into the conditioned media as a latent homodimer that regains full biological activity after treatment with reducing agents (*36*, *37*). The fact that we observed both human C131S and bovine S131C still able to form homodimers (although the amount is decreased relative to the wild-type proteins) upon treatment with $CuCl_2$ treatment argues that the two cysteine residues conserved between the two proteins may be important for this novel secretion method. Indeed, it has been shown that one of these conserved cysteines (cysteine-30) is critical for FGF-1 release in response to heat shock (*40*).

In conclusion, we have shown that the abrogation of heparin affinity of iodinated human FGF-1 can be reverted by substituting the cysteine residue at position 131 with serine. The opposite effect is observed with bovine FGF-1 that has the serine at position 131 changed to cysteine. These results confirm the previous reports for the putative heparin-binding domain in FGF-1 and extend the observation in showing that cysteine-131 contributes to such a binding. Under the oxidative conditions during iodination, the cysteine residue at position 131 (in wild-type human and bovine S131C mutant FGF-1 proteins but not in human C131S and wild-type bovine and newt FGF-1 proteins) is oxidized sequentially to cysteic acid. This modified residue possesses a negative charge which could disrupt the interaction between the negative charge on heparin or heparan sulfate and the net positive charge in this region of FGF-1. However, residue 131 is 11−12 Å distal to the bound sulfate in the structure of human FGF-1 (*29*). The structure of bovine FGF-1 with a heparin analogue, sucrose octasulfate, indicates that this molecule is 9−10 Å distal to the side chain of residue 131 (*28*). Thus, a like-charge repulsion of negatively charged heparin by cysteic acid through electrostatic interaction alone would seem unlikely. An alternative explanation is that the oxidation of cysteine-131 to cysteic acid destabilizes the FGF-1 structure to the extent that it is unfolded. Computer modeling of the molecular structures of both native FGF-1 and iodinated FGF-1 as shown in Figure 10 excluded this possibility. Indeed, we were able to show that the negatively charged cysteic acid at position 131 attracted lysine-126 and lysine-132 such that the latter are no longer available to interact with the negatively charged sulfated groups of heparin (Figure 10).

The difference in heparin affinity between the two iodinated wild-type FGF-1 proteins correlates with the observation that bovine FGF-1 is less dependent on exogenous heparin for optimal mitogenic activity (*31*, *49*). In the absence of reducing agents, FGF-1 has been demonstrated to undergo rapid, proteolytic-independent inactivation (*34*). This inactivation has been attributed to the formation of intra- or intermolecular disulfide bonds or the formation of mixed thiols. Our data suggest that the oxidative inactivation may also involve the prevention of an FGF-1−heparin complex. Since heparin is essential for FGF-1 biological activity, blocking their interaction would lead to FGF-1 inactivation. This suggests that cysteine-131 has important implications in the regulation of heparin binding by FGF-1 and its subsequent activity. Moreover, the increased recovery (>20-fold) from heparin−agarose of iodinated human FGF-1 afforded by the C131S mutant provides a valuable reagent needed in the many assays that utilize this potent growth factor.

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