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Probing the role of Proline -135 on the structure, stability, and cell proliferation activity of Human Acidic Fibroblast Growth Factor.

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Abstract

Human acidic fibroblast growth factor 1 (hFGF1) is a protein intricately involved in cell growth and tissue repair. In this study, we investigate the effect(s) of understanding the role of a conserved proline (P135), located in the heparin binding pocket, on the structure, stability, heparin binding affinity, and cell proliferation activity of hFGF1. Substitution of proline-135 with a positively charged lysine (P135K) resulted in partial destabilization of the protein; however, the overall structural integrity of the protein was maintained upon substitution of proline-135 with either a negative charge (P135E) or a polar amino acid (P135Q). Interestingly, upon heparin binding, an increase in thermal stability equivalent to that of wt-hFGF1 was observed when P135 was replaced with a positive (P135K) or a negative charge (P135E), or with a polar amino acid (P135Q). Surprisingly, introduction of negative charge in the heparin-binding pocket at position 135 (P135E) increased hFGF1's affinity for heparin by 3-fold, while the P135K mutation, did not alter the heparin-binding affinity. However, the enhanced heparin-binding affinity of mutant P135E did not translate to an increase in cell proliferation activity. Interestingly, the P135K and P135E double mutations, P135K/R136E and P135/R136E, reduced the heparin binding affinity by ~3-fold. Furthermore, the cell proliferation activity was increased when the charge reversal mutation R136E was paired with both P135E (P135E/R136E) and P135K (P135K/R136E). Overall, the results of this study, suggest that while heparin is useful for stabilizing hFGF1 on the cell surface, this interaction is not mandatory for activation of the FGF receptor.

Keywords

fibroblast growth factor; heparin binding; charge-reversal; bioactivity; stability

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Introduction

hFGF1 is a member of a family of polypeptides recognized as powerful mitogens which are involved in cell proliferation, cell differentiation, and wound healing processes [1–5]. FGFs initiate these processes through activation of the tyrosine kinase cell surface receptors (FGFRs). [5–7]. Unlike other family members, human acidic fibroblast growth factor (hFGF1) binds to all four isoforms of FGFRs, and therefore it is an ideal target for therapeutic applications [4, 5, 8, 9]. The major caveat in using hFGF1 as a wound-healing agent is its poor inherent stability, which significantly shortens its bioavailability. In fact, a significant population of hFGF1 is known to exist in denatured state(s) under physiological temperatures [10–12].

Binding of hFGF1 to the heavily sulfated cell surface glycosaminoglycan, heparin, is known to confer structural stability leading to its increased physiological half-life [13–15]. Heparin binding occurs at the c-terminal domain of hFGF1 to a cluster of positively charged residues recognized as the heparin binding pocket [16]. Although there is a general agreement that heparin stabilizes hFGF1, the exact physiological role of heparin in hFGF1 mediated signaling is still a subject of debate. Heparin is widely believed to be mandatory for hFGF1 signaling. Crystal structures of the FGF-FGFR- dimeric complexes show that both hFGF1 and its FGFR appear to be associated through their interactions with heparin [17–21]. Site-directed mutagenesis studies have identified a number of mutations that stabilize hFGF1. Furthermore, some of these stable hFGF1 mutants have been shown to exhibit increased levels of cellular proliferation activity in the absence of heparin [22]. In another study, it has been shown that a charge reversal mutation, K132E, not only diminished heparin binding affinity but also decreased the mitogenic activity of hFGF1. These studies suggest that heparin binding *per se* is not critical for the activation of the cell surface receptor of hFGF1 [23].

hFGF1 has been shown to be susceptible to thrombin action. The proteolytic enzyme has been shown to render hFGF1 inactive by specifically cleaving hFGF1 at the secondary cleavage site, R136 [24]. This aspect has been a significant bottleneck for FGF-based wound care therapeutics because both thrombin and FGF are known to be simultaneously present at a wound site [24, 25]. In this context, we recently studied the effect(s) of a charge reversal mutation at position R136 on the structure, stability, heparin binding affinity and cell proliferation activity of hFGF1. Interestingly, the single point charge-reversal mutation, R136E, was found to reduce hFGF1's affinity to heparin while significantly increasing its cell proliferation activity [26]. In this context, here we examine if similar effects on the structure, stability, and cell proliferation activity can be achieved through substitution of the conserved proline-135 with charged residues.

Proline-135 (P135), located in the heparin-binding pocket, is well conserved among the FGF1 family. Previously, using biophysical and molecular dynamics studies we showed that P135G mutation caused a subtle change in the solvent-exposed non-polar surfaces in the protein but it significantly increased the susceptibility of hFGF1 to trypsin protease [16]. In addition, it was found that P135G mutation resulted in the decrease in membrane

permeability of hFGF1 and consequently the stress-induced release of the growth factor was significantly affected. In this background, we examined the role of P135 on the stability, heparin-binding affinity, and cell proliferation activity of hFGF1. Results of this study for the first time showed that P135 alone and in conjunction with R136 contributes significantly to the heparin structure and bioactivity of hFGF1. In addition, the findings of this study demonstrate that heparin contributes to the stability of hFGF1 but the cell proliferation activity of the growth factor is not strictly dependent on its affinity to bind to heparin.

Results and Discussion

Conservation and spatial microenvironment Proline135 in the structure of hFGF1

The three-dimensional structure of the heparin – hFGF1 binary complex reveals that the c-terminal domain (residues, N120-H138) of the protein plays an important role in heparin binding [19, 27, 28]. hFGF1 to heparin through electrostatic interactions with several positively charged residues (K126, K127, K132, R133, R136) located in this heparin-binding pocket [27]. These positively charged residues are primarily located in the flexible loops between beta strands X, XI, and XII. K127, K132, G134, and R136 are well conserved among different isoforms of FGF. Interestingly, alignment of amino acid sequences of FGF1 isolated from different species shows that the residues in the heparin-binding pocket, including residues 132 to 137, are highly conserved. The well-conserved P135 is located in the loop connecting β -strands XI and XII. P135 is positioned in the center of a triangle with the three positively charged residues (K119, R133 and R136) constituting the three corners of the triangle (Fig 1). Crystal structure of the heparin-hFGF1 binary complex (PDB 2ERM) shows that the C α atom of P135 is positioned within 4 - 7 Å of the C α atom(s) of K119, R133 and R136 [27, 28]. The structural rigidity imparted by P135 is believed to be critical for the orientation of the side-chains of the positively charged residues (R133, and R136) to optimally interact with the negatively charged heparin.

Mutations at position 135 modestly perturb the tertiary structure of hFGF1.

Wt-hFGF1 and all the designed mutants were purified to homogeneity using heparin Sepharose affinity chromatography (Fig S1). Except for the double mutant, P135E/R136E, all the other mutants eluted in high salt concentration (1500 mM NaCl) similar to wt-hFGF1. P135E/R136E eluted in 500 mM NaCl, suggesting decreased heparin binding affinity. The Far-UV circular dichroism (CD) spectra of the designed mutants of hFGF1 overlaid well with the wild type, exhibiting the characteristic positive ellipticity band in the wavelength range 220 - 240 nm, and a negative band in the region of 200 - 210 nm (Fig. 2). These spectral features indicate that the native β -trefoil conformation is not significantly perturbed due to the designed mutations (Fig. 2).

Wt-hFGF1 contains eight tyrosine residues and a lone tryptophan residue (W121). The intrinsic fluorescence spectrum of native wt-hFGF1 conspicuously shows an emission maxima (at 308 nm) representative of the tyrosine fluorescence. The fluorescence of the single tryptophan residue (W121) is mostly quenched by lysine and proline residues that are located in the spatial vicinity of W121 [29]. However, in the denatured state(s) of hFGF1, the positioning of the indole ring of W121 is shifted away from the amino groups, which are

known to quench W121 fluorescence in the native conformation of hFGF1. Therefore, the denatured state(s) of hFGF1 is manifested by the appearance of the tryptophan fluorescence around 350 nm. Similar to wt-hFGF1, the intrinsic fluorescence spectra of all the designed mutants of hFGF1 showed the characteristic tyrosine emission maximum at 308 nm (Fig. 3). However, unlike wt-hFGF1, all designed hFGF1 P135 single mutants, and the P135/R136 double mutants exhibit a broad shoulder in the 350 nm region. The indole side chain of W121 is positioned ~ 3.5 Å from the pyrrole side chain of P135, therefore introduction of a lysine, glutamine, or glutamate at position 135 plausibly induces a minor change in the local microenvironment, which causes the drift of the indole ring of W121 to move away from the quenching groups. Interestingly, the relative fluorescence intensity of W121 was slightly different for each hFGF1 mutant, which can be attributed to local structural perturbations. In the single mutants, P135E and P135Q, the 350 nm fluorescence intensity was moderately increased, but the ratio of tyrosine fluorescence at 308 nm to tryptophan fluorescence at 350 nm was still high (Fig. S2). However, the 350 nm emission intensity of the P135K variant was greater than for any other hFGF1 mutant and the 308/350 nm fluorescence ratio was diminished. This indicates that introduction of a positively charged lysine at position 135 may be generating charge repulsion with spatially close positively charged residues, K119, R133, and R136, causing significant perturbation of the indole ring of W121. The fluorescence spectrum of double mutant, P135E/R136E, revealed that the 350 nm emission intensity is modestly increased in a similar manner as observed in the case of the P135E and P135Q mutants. Interestingly, the fluorescence spectrum of the double mutant, P135K/R136E, is most similar to wt-hFGF1. These results indicate that introduction of a negatively charged residue (via the R136E mutation) appears to nullify the destabilizing effects(s) of the P135K mutation. In summary, the results of the intrinsic fluorescence and far UV CD data, analyzed in conjunction, suggest that the designed mutations cause minor tertiary structural changes but do not significantly perturb the backbone conformation of hFGF1.

Introduction of positive charge at position 135 increases the structural flexibility of hFGF1

Anilino naphthalene 8- sulfonate (ANS) binding is commonly used to examine the tertiary structural changes in proteins [30]. In this context, we measured the changes in the ANS fluorescence to examine the structural perturbation(s) caused by mutations introduced at position 135. ANS is an extrinsic fluorescent probe that binds to solvent-accessible hydrophobic surface(s) in proteins [30]. As hydrophobic residues are typically buried in the protein core, increase in ANS fluorescence is suggestive of greater solvent-accessible hydrophobic surface(s). The ANS binding curves of mutants P135E, P135E/R136E, P135Q, and P135K/R136E are quite similar to wt-hFGF1, indicating the tertiary folding of the hFGF1 does not significantly change due to the introduced mutations (Fig. 4). However, the relative emission intensity of ANS (at 500 nm) upon binding to the P135K mutant is about two-fold higher than when bound to wt-hFGF1, indicating that introduction of positive charge at position 135 induces a modest conformational change resulting in an increase in the solvent-exposure of the hydrophobic surface(s). These results corroborate well with those obtained based on the changes in the intrinsic tryptophan fluorescence

Trypsin, a serine protease, cleaves proteins at the c-terminal end of lysine and arginine residues. Therefore, limited trypsin digestion (LTD) assay is a useful technique to monitor

the subtle changes in flexibility of the backbone caused by introduction of the mutations in the heparin-binding pocket of hFGF1 [31], We performed LTD assay on wt-hFGF1 and the designed mutants, in the presence and absence of heparin, to determine the effect(s) of the individual mutations on the conformational flexibility of hFGF1. Examination of the rate of digestion of wt-hFGF1 and the designed mutants by trypsin, in the absence of heparin, showed that after 40 minutes incubation with the enzyme, wt-hFGF1 and the P135E/R136E mutant are digested by 10% and 20% respectively (Fig. 5 and Fig. S3). Unlike wt-hFGF1, the P135E, P135K/R136E, and P135Q mutants showed higher susceptibility to trypsin. Their original band intensity, after 40 minutes incubation with trypsin, decreased by ~50%, ~60%, and ~75% respectively (Fig. 5A). Introduction of positive charge at position 135 drastically increases the susceptibility of hFGF1 to trypsin degradation, as interestingly, the P135K mutant is 80% digested after the first 10 minutes and is completely digested within 40 minutes exposure to trypsin (Fig. 5 and Fig. S3). These results suggest that the introduction of an extra positive charge, via the P135K mutation, appears to enhance the flexibility of the backbone due to increased charge repulsions between the cluster of positively charged residues located in the heparin-binding pocket. Alternatively, it may be argued that the increased trypsin susceptibility of the P135K mutant is due to introduction of an additional trypsin cleavage site. However, this possibility is unlikely because the double mutant, P135K/R136E is relatively more resistant to the action of trypsin than the P135K mutant. The lower trypsin susceptibility of the double mutant, P135K/R136E, as compared to the single mutant, P135K, appears to suggest that the negative charge introduced at position 136 partially nullifies the enhanced repulsions in the heparin binding pocket caused by the addition of an extra positive charge via the P135K mutation. In summary, from analysis of the data presented so far, it appears that introduction of negative charge at position P135, individually and in tandem with mutation R136E does not seem to alter the backbone conformation of the protein but in fact appears to render the tertiary structure of hFGF1 more compact.

Mutations at position 135 only cause local structural changes

^1H - ^{15}N Heteronuclear single quantum coherence (HSQC) spectroscopy is a two-dimensional NMR technique commonly employed to monitor atomic-level changes in the backbone conformation of proteins. Superimposition of the ^1H - ^{15}N HSQC spectra of P135K on wt-hFGF1 and analysis of the ^1H - ^{15}N chemical shift perturbation plot (Fig. 6A & B) indicated that introduction of positive charge at position 135 induces drastic shift in the crosspeak corresponding to G134, which is located in the heparin-binding pocket. In addition, residues T83 and G85, located in the disordered loop, which is placed ~6 Å from P135, were significantly perturbed. Similarly, analysis of the chemical shift perturbation data of the P135E mutant also revealed that residues located in the heparin-binding pocket (G134, R133, R136, and Y139) expectedly showed significant ^1H - ^{15}N chemical shift perturbation (Fig. 7A & B). Additionally, residues G85 and T83, which are in spatial proximity to position 135, are also significantly perturbed. The ^1H - ^{15}N chemical shift perturbation observed for R133 and R136, upon introduction of negative charge at position 135 (P135E), may perhaps be due to the formation of favorable electrostatic interactions that plausibly reorient the positioning of these critical heparin-binding residues. Superimposition of the ^1H - ^{15}N HSQC spectra of the double mutant P135E/R136E on that of wt-hFGF1 showed that

G134 and R136 are the predominantly perturbed residues within the heparin-binding region. T83 and G85 are the most significantly perturbed residues outside of the ligand-binding pocket (Fig. S4 A & B). Overlay of the ^1H - ^{15}N HSQC spectra of P135K/R136E with that of wt-hFGF1 indicated that the backbone folding of the protein is quite similar to wt-hFGF1 (Fig. S5 A & B). With the exception of L87 (which is located on beta strand XII in spatial proximity to position 135), the ^1H - ^{15}N crosspeaks of most other residues in the protein showed minimal or no perturbation. The multidimensional NMR data on the P135K/R136E double mutant suggest that introduction of a negative charge at position 136 helps to nullify the destabilizing interactions that come into play due to the introduction of an extra positive charge in the heparin binding pocket through the P135K mutation. These results are quite consistent with the conclusions drawn from the intrinsic fluorescence, ANS binding, and LTD assays, which suggest that the structural integrity of the protein is maintained.

Introduction of negative charge at position 135 and/or 136 introduces additional salt bridges and hydrogen bonding in the heparin-binding region of hFGF1.

Molecular dynamics simulations were performed on each of the designed mutants of hFGF1 to gather support for the conclusions drawn from the biophysical experiments. Simulations were performed using the crystal structure of wt-hFGF1 in the absence of heparin (Protein Data Bank code 1RG8). Movies of the simulations are provided in the supporting information (supplemental movie S1, S2, S3, S4, S5, and S6 respectively). From each trajectory, the inter-domain distances of the C α backbone atoms were measured as a function of time, showing no major differences between wt-hFGF1 and all the hFGF1 mutants (Fig. S6 A). Additionally, a comparison of the root mean square fluctuations (RMSF) of the C α atoms of each mutant to wt-hFGF1 (Fig. S6 B) indicated no significant differences in the RMSF of mutant P135K from wt-hFGF1. The RMSF for mutant P135Q is decreased compared to wt-hFGF1 for certain residues between positions 30 to 80, corresponding to the flexible loop regions of the protein (Fig. S6 B). RMSF of P135E is modestly increased for residue L27, located on beta strand I, residue R49 located on the flexible loop region between beta strands III and IV, and residue K115 situated within the heparin-binding region. RMSF of double mutant P135E/R136E is modestly lower for residue Q59 and residues E67-Y69 found within beta strands IV and V, respectively. However, the RMSF is significantly higher for loop regions containing M81 and K115. The RMSF for the double mutant, P135K/R136E, is significantly higher for residues L27, L58, Y69, which are located on beta strand I, IV, and V respectively.

Salt bridge and hydrogen bonding analyses were also performed on wt-hFGF1 and each of the designed mutants. In the molecular dynamics simulation of P135K, it was observed that the side chains of R133, K135, and R136 are oriented in relatively opposite directions (Fig. S7). The guanidinium head groups of the arginine residues were positioned away from each other, plausibly to minimize steric hindrance and destabilizing electrostatic repulsion(s). Overall, P135K had one fewer salt bridge than wt-hFGF1. Additionally, the P135K mutant contained no hydrogen bonds or salt bridges involving residues R133, K135, and R136 with any other residues located in the spatial vicinity of the heparin-binding region (Table S1). The absence of any additional stabilizing interactions in the heparin-binding region is also

supported by the fluorescence, ANS binding, trypsin digestion, and equilibrium unfolding data.

Similar to the P135K mutant, the P135Q mutant had only one less salt bridge than wt-hFGF1 (Table S1). Hydrogen bonding analysis revealed the presence of stabilizing hydrogen bonds between the side chain amide group of Q135 and the carbonyl group on the backbone of D84 (Fig. S7). In addition, hydrogen bonding also occurs between the carbonyl group on the side chain of Q135 and the amide group in the backbone of Q135. Substitution of proline with glutamine at position 135 did not induce stabilizing interactions with residues R136 or R133, which would potentially leave these two critical heparin-binding residues available for interaction with heparin. Therefore, these observations are consistent with the conclusions drawn from the structure and stability data, which suggest that P135Q does not significantly alter the protein stability or interaction(s) with heparin.

Hydrogen bonding analysis of double mutant P135K/R136E revealed stabilizing bonds between the side chain amine group of K135 and the side chain carboxyl group of D84. The salt bridge pattern in the double mutant, P135K/R136E, is identical to wt-hFGF1, which lends support to the conclusions drawn from equilibrium unfolding, ANS, and intrinsic fluorescence experiments. Interestingly, no salt bridge was observed to form between K135 and E136. However, E136 was found to be neutralized by a salt bridge formed between E136 and R133 (Fig S7). Hydrogen bonding was also observed between the side-chain carboxyl group of E136 and the guanidium head group of the side chain of R133 as well as between the backbone amide group of E136 and the backbone carbonyl group of R133. These interactions between R133 and E136 appear to stabilize hFGF1 and diminish the affinity hFGF1 for heparin.

Furthermore, one additional salt bridge in the heparin-binding pocket was observed in the P135E mutant between residues E135 and R133. Several additional salt bridges were observed in the double mutant, P135E/R136E, between residues, E135 - R133, E136 - K132, and E136 - R133 (Table S1). For mutant P135E, the interaction between E135 and R133 plausibly reduces contact between R133 and heparin. These conclusions are consistent with the thermal stability data of the heparin-bound P135E which showed a $\sim 10^\circ$ lower stability than heparin-bound wt-hFGF1 (Table 1). Additionally, the multiple salt bridges formed within the heparin-binding region of P135E/R136E may increase the native protein's overall stability while diminishing the heparin-binding affinity. These conclusions are consistent with the thermal stability data of the P135E/R136E mutant (Table 1).

Heparin binding increases the thermal stability of P135K but not of other hFGF1 mutations

The thermal stability of hFGF1 and the designed mutants, was measured by Far UV CD spectroscopy by monitoring ellipticity changes (at 228 nm). Analysis of the denaturation temperature, T_m (the temperature at which 50% of the protein population exists in the denatured state(s)), revealed that in the absence of heparin, all the hFGF1 mutants except the double mutants, P135E/R136E ($T_m = 53^\circ\text{C} \pm 0.87$) and P135K/R136E ($T_m = 48.5^\circ\text{C} \pm 0.91$), exhibited a marginally lower thermal stability than wt-hFGF1 ($T_m = 48.5^\circ\text{C} \pm 0.72$) (Fig. 8A) (Table 1). In the presence of heparin, all the designed hFGF1 mutants are less stable than wt-hFGF1. Interestingly, heparin binding increased the thermal stability of

P135K ($T_m = 20.8^\circ\text{C}$) to the same extent as wt-hFGF1 ($T_m = 20.5^\circ\text{C}$). However, introduction of a negative charge (P135E) or a polar functional group (P135Q) at position 135 modestly reduced the net increase in T_m upon binding to heparin [(P135E $T_m = 15.3^\circ\text{C}$) and (P135Q $T_m = 16.2^\circ\text{C}$)] (Fig. 8A). The total increase in T_m value of P135E/R136E upon binding to heparin was only very modest $\sim 4.7^\circ\text{C}$. The T_m of the P135K/R136E mutant was not found to increase upon binding to heparin (Table 1). The insignificant or no increase in stability upon binding to heparin for both the double mutants (P135E/R136E and P135K/R136E) suggests that the charge reversal at position 136 has a unique effect of diminishing heparin binding affinity of the protein.

The stability of each mutant, in the absence and presence of heparin, was also measured by urea-induced denaturation (Fig. 8B). In the absence of heparin, for all the designed hFGF1 mutations with the exception of the double mutants, (P135E/R136E ($C_m = 1.8\text{M} \pm 0.67$) and P135K/R136E ($C_m = 1.8\text{M} \pm 0.26$)), the C_m values (concentration of the denaturant at which 50% of the protein population is in the denatured state(s)) were lower than that of the wt-hFGF1 ($C_m = 1.80\text{M} \pm 0.11$) (Table 1). Interestingly, in the presence of heparin, P135E ($C_m = 2.6\text{M}$) was stabilized to the same extent as wt-hFGF1. Heparin did not stabilize any of the other designed mutants to the extent it stabilized wt-hFGF1 and P135E. Of the hFGF1 single mutants, P135K and P135Q were stabilized by heparin the least (Table 1 and Fig. 8B). Lastly, the C_m value(s) for the double mutants, P135E/R136E ($C_m = 0.5\text{M}$) and P135K/R136E ($C_m = 0.9\text{M}$) were significantly reduced compared to wt-hFGF1. The lack of heparin-induced stabilization for both double mutants is likely due to the additional stabilizing salt bridges formed within the heparin-binding region previously discussed in the MDS section.

Overall, the thermal and urea equilibrium unfolding data indicate that substitution of proline at position 135 with lysine or glutamine modestly reduced the ability of heparin to stabilize these mutants compared to wt-hFGF1. This may be due to a decrease in structural stability, which is observed from the fluorescence and trypsin digestion data. Furthermore, the presence of the charge reversal mutation at position 136 (R136E) significantly diminished the stabilizing effect(s) of heparin toward hFGF1, as observed with the double mutants P135K/R136E and P135E/R136E (Table 1). These conclusions are in good agreement with the conclusions drawn based on the thermal denaturation data.

Introduction of two negative charges in the heparin-binding pocket reduces heparin-binding affinity

Isothermal titration calorimetry (ITC) is a useful technique for the characterization of the thermodynamics of ligand-protein interactions. Comparison of the apparent binding affinity [$K_d(\text{app})$] values, representing the interaction of wild type hFGF1 and the P135K mutant with heparin, showed that introduction of positive charge at position 135 does not have any significant effect(s) on the heparin-binding affinity, [P135K $K_d(\text{app}) = 1.58\text{ }\mu\text{M}$, Fig. 9]. In contrast, introduction of negative charge at position 135 (P135E) increased the heparin-binding affinity by three-folds [P135E $K_d(\text{app}) = 0.58\text{ }\mu\text{M}$, Fig. 9]. It was originally predicted that introduction of a positive charge at position 135 would increase binding affinity of hFGF1 for heparin and that introduction of a negative charge would likely reduce

the affinity for the negatively charged heparin. However, it appears that the side chain of E and K, at position 135, are oriented away from the hFGF1-heparin binding interface because mutations at this site (P135) did not show the anticipated effect(s) on heparin binding. Introduction of a polar functional group at position 135 modestly increased the heparin-binding affinity of hFGF1 [P135Q $K_{d(app)} = 1.02 \mu\text{M}$ Fig. 9], which could be due to the slightly more compact structure of the mutant compared to wt-hFGF1. This aspect is consistent with the ANS binding and molecular dynamics data. Introduction of negative charge at combined positions 135 and 136 reduced the affinity of hFGF1 for heparin as expected [P135E/R136E $K_{d(app)} = 4.65 \mu\text{M}$, Fig 9]. Therefore, it appears that the addition of the second negative charge at position 136 drastically reduces the affinity of the protein to heparin. These results are in good agreement with the thermal and urea denaturation data. Finally, the double mutant, P135K/R136E, was also found to exhibit reduced binding affinity for heparin [P135K/R136E $K_{d(app)} = 5.56 \mu\text{M}$, Fig. 9] despite introduction of a positive charge at position 135 (Fig. 9). As the single mutant, P135K, did not show increased binding affinity to heparin, the observed reduction in heparin-binding affinity of the double mutant P135K/R136E appears to be largely due to the charge reversal mutation at position 136 (R136E).

ITC data provides valuable information on the thermodynamics governing protein-ligand interactions as well [14]. The change in enthalpy (ΔH) represents interactions including electrostatic, hydrogen bonding, and van der Waals [14]. The change in entropy (ΔS) represents changes in solvation as well as conformational changes within the protein upon binding with heparin [14]. The enthalpy values characterizing the interaction of heparin with the designed mutants [P135E ($\Delta H = -1.11 \pm 0.2 \text{ k.cal mol}^{-1}$), P135Q ($\Delta H = -1.08 \pm 0.6 \text{ kcal mol}^{-1}$), and P135K/R136E ($\Delta H = -1.04 \pm 0.73 \text{ kcal mol}^{-1}$)] of hFGF1 were about half of that compared to the ΔH values representing the wt-hFGF1-heparin interaction ($\Delta H = -2.14 \pm 0.4 \text{ k.cal mol}^{-1}$). These results suggest that the degree of contact between the designed hFGF1 mutants and heparin is reduced (Table S2). Additionally, the change in enthalpy observed for the interaction of heparin individually with P135K ($\Delta H = -0.85 \pm 0.2 \text{ k.cal mol}^{-1}$) and P135E/R136E ($\Delta H = -0.85 \pm 0.5 \text{ k.cal mol}^{-1}$) were approximately three-fold lower than the wt-hFGF1-heparin interaction, which indicates that the degree of contact between these mutants and heparin is significantly reduced (Table S2). The $T\Delta S$ values for the interaction of heparin with the individual hFGF1 mutants are five- and ten-fold lower as compared to the interaction of the glycosaminoglycan with wt-hFGF1 ($T\Delta S = -1.1 \text{ k.cal mol}^{-1}$). These results indicate that any conformational change(s) or desolvation occurring at the binding interface of the individual hFGF1 mutants, upon interaction with heparin, are minimized (Table S2). However, despite an increase in the entropic term, all entropic values are still negative and therefore favorable.

Binding affinity does not positively correlate with mitogenic activity

It is generally believed that heparin plays a critical role in the interaction of hFGF1 with its receptor. By this premise, it is likely that an increase in the binding affinity of hFGF1 to heparin should likely correlate with an increase in the growth factor-mediated mitogenic activity. If so, it can be expected that hFGF1 mutants that exhibit higher binding affinity to heparin than wt-hFGF1 would exhibit enhanced cell proliferation activity. In this context, we

measured the proliferation of heparinase-treated NIH3T3 cells by wt-hFGF1 and the designed mutants in the presence and absence of heparin (Fig. 10). Maximum cell proliferation with wt-hFGF1 was achieved at a heparin to protein ratio of 10:1, and therefore all cell proliferation assays were performed at this ratio. Fig. 10A shows that in the absence of heparin, mutants P135K and P135E exhibit modestly higher cell proliferation activity than wt-hFGF1. However, in the presence of heparin, both P135K and P135E showed slightly lower activity than wt-hFGF1 (Fig. 10B). Reduced cell proliferation activity of P135K is perhaps due to the decreased structural stability ascribed to the protein as measured by fluorescence, LTD, and equilibrium unfolding experiments. Interestingly, the structural integrity of the P135E mutant is maintained as measured by fluorescence, LTD, and equilibrium unfolding experiments. In addition, the heparin-binding affinity of P135E is ~ 3-times higher than that of wt-hFGF1 but yet the cell proliferation activity of this mutant is lower than that exhibited by wt-hFGF1. These results suggest that the mitogenic activity of hFGF1 is not strongly correlated to its binding affinity to heparin. In addition, Figures 10 A & B indicate that both double mutants, P135E/R136E and P135K/R136E, in the presence and absence of heparin, exhibit higher cell proliferation activity than wt-hFGF1. As previously mentioned, ITC data suggests that both double mutants, P135E/R136E and P135K/R136E, have no or insignificant affinity to bind to heparin and the loss of heparin binding affinity is likely to be strongly associated with the introduction of a negative charge at position 136. Overall, the results of the cell proliferation experiments clearly indicate that the heparin binding is not a prerequisite for the mitogenic activity of hFGF1.

The notion that heparin is not mandatory for hFGF1 activation of cell surface receptors has been previously reported [10, 12, 32–34]. Wong et al (1995) showed that charge reversal K132E did not alter the cell proliferation activity of hFGF1 despite its reduced affinity to heparin [35]. Similarly, Culajay et al (2000) demonstrated that substitution of the three cysteine residues with serine decreased heparin-binding affinity of hFGF1. Additionally, the cysteine to serine substitution(s) increased the physiological half-life of hFGF1 and also enhanced the cell proliferation activity of hFGF1 [12]. Furthermore, combination of mutations L58F, H35Y, H116Y, and F122Y revealed an increased thermal stability, even in the absence of heparin, without any significant loss in the cell proliferation activity of hFGF1 [10]. Inclusion of mutations at additional sites on the quadruple mutant (L58F/H35Y/H116Y/F122Y) to generate a septuplet mutant (H35Y/Q54P/L58F/S61I/H107G/H116Y/F122Y) increased the stability of hFGF1 significantly [32]. Interestingly, the septuplet mutant was also found to exhibit six-fold higher cell proliferation activity than the wildtype protein in the absence of heparin [32]. In this context, the results of this study clearly show that heparin is not a pre-requisite for the cell proliferation activity of hFGF1. Heparin, present on the cell surface, plausibly serves as a reservoir to facilitate the accumulation of hFGF1 on the cell surface and also to increase the stability of the growth factor through electrostatic interactions.

Conclusions

An understanding of the structure-function relationship between hFGF1 and heparin is important for the design and development of FGF-based therapeutics for wound healing and tissue regeneration applications. The data obtained in this study suggest that the stability and

heparin binding affinity of hFGF1 can be modulated by mutations in the heparin-binding pocket. The results of this study suggest that the primary role of heparin is to confer structural stability to hFGF1. In addition, the results obtained in this study conclusively suggest that increased affinity for heparin does not necessarily result in increased cell proliferation activity.

Materials and Methods

Materials:

DNA plasmid isolation kits were purchased from Qiagen, USA and Quikchange II XL mutagenesis kits were obtained from Agilent. Competent cells (DH5 α and BL-21(DE3)) were sourced from Novagen Inc., USA. Lysogeny broth (LB) was obtained from EMD Millipore, USA. Heparin sepharose was obtained from GE Healthcare, USA. VWR Scientific Inc, USA was the supplier for all buffer components including Na₂HPO₄, NaH₂PO₄, NaCl, and (NH₄)SO₄. Low molecular weight heparin sodium salt (~3kDa) was procured from Sigma and MP Biomedicals LLC. NIH 3T3 cells were sourced from American Type Culture Collection (ATCC) and additional cell culture reagents such as, DMEM media, fetal bovine serum (FBS), and penicillin streptomycin were obtained from Thermo Fisher Scientific USA.

Molecular Dynamic Simulations:

hFGF1 crystal structure (PDB 1RG8) was visualized using the Pymol visualizing software. All mutations were created using Pymol mutagenesis tool [36]. Structures were first energy-minimized for 2000 steps and then immediately solvated in a water box of 12 $\times$ 12 $\times$ 12 Å³. 0.150 M NaCl was then added to neutralize the solvent box. Following solvation, the solvent molecules and ions as well as the protein backbone and side chains were relaxed while hydrogen atoms were kept rigid. Relaxation was performed to prepare the system for equilibration. Equilibration of the system was implemented in the NPT ensemble utilizing the CHARMM36 force field and NAMD 2.9 [37, 38]. Side-chains were relaxed for 10 picoseconds (ps) with the backbone fixed in the absence of solvent molecules. Subsequently, water molecules were relaxed for 1500 steps while the protein atoms remained fixed. Relaxation of the system was followed by 50 ps of dynamics. Finally, the solvent molecules were relaxed around the protein with harmonic constraints using a force constant of 1 kcal/(mol Å²). After relaxation of the solvent molecules, the temperature of the system was increased in small increments (10 K per 2 ps) until a final temperature of 300 K was reached. Temperature adjustment was followed by 250 ps of dynamics. Temperature (300 K) was sustained by Langevin dynamics with a damping coefficient of 1 ps⁻¹ and a pressure of 1 atmosphere with a 100 femtosecond (fs) period, and 50 fs decay time was sustained by the Langevin piston method [39]. Long-distance electrostatic interactions were determined using particle mesh Ewald (PME) method and periodic boundary conditions. Additionally, a switching function was applied to terminate electrostatic and Van der Waals interactions greater than 12 Å. Production runs of 100 nanoseconds (ns) were performed and protein stability was evaluated from the resulting simulations. Visual Molecular Dynamics (VMD) 1.9 was used to visualize the simulation(s) and to trace hydrogen bonding of the mutated residue [40].

Construction and purification of hFGF1 mutants:

For all site-directed mutagenesis, a form of hFGF1 without the first 14 N terminal residues (residues 15-154) was used. This 14-amino acid N-terminal segment is unstructured and has been well documented in the literature to be not important for the structure and mitogenic activity of hFGF1 [17, 33]. pET20b bacterial expression vector was the template for the site-directed mutagenesis. Agilent primer design software was used to design the desired primers, which were ordered from IDT DNA Inc., USA. Instructions provided by the manufacturer for the QuikChange II XL kit were followed to conduct site-directed mutagenesis (SDM). The heat shock technique was used to transform the plasmid into DH5 α competent cells and the sequence of the plasmid was verified by the University of Arkansas Medical Science – DNA core sequencing facility [41]. Once the correct sequence was verified, overexpression of the wt-hFGF1 and designed mutants was achieved using BL-21(DE3) *Escherichia coli* host cells. Bacterial cells were incubated overnight at 250 rpm and 37°C in LB. It is recognized that hFGF1 overexpressed in *E. coli* lacks post-translational modification; however, no post-translational modification(s) have been reported to occur in hFGF1. Following overexpression, bacterial cells were lysed *via* ultra-sonication and the released proteins were separated from the cell debris by centrifugation for 1 hour at 19,000 rpm. Purification of the hFGF1 mutants was accomplished using heparin sepharose resin. Purification of hFGF1 was achieved using a stepwise NaCl gradient in 10 mM sodium phosphate buffer (PB) containing 25 mM (NH₄)₂SO₄ at pH 7.2 according to methods described previously [42]. Pure hFGF1 protein was typically obtained in the 1500 mM NaCl buffer fraction [10, 32, 33]. Protein purity was analyzed using SDS-PAGE. Apparent molecular mass of the purified hFGF1 samples was compared against a pre-stained standard protein molecular mass marker ranging from 7 kDa –175 kDa. Protein concentrations were determined by Bradford assay using a Hitachi F-2500 fluorimeter.

Heparin-binding affinity of hFGF1 mutants:

Isothermal titration calorimetry (iTC-200, Malvern Inc.) was employed to determine the apparent hFGF1-heparin binding affinity. Heat changes were measured by titrating heparin (loaded in the syringe) into protein solution in the reaction vessel. All protein and heparin samples were made in solution containing in 10 mM phosphate buffer with 100 mM NaCl and 25 mM (NH₄)₂SO₄ (pH 7.2) and were degassed prior to titration. A protein concentration of 50 μ M and a heparin concentration of 500 μ M was consistently used to maintain a 1:10 ratio of protein to heparin in all ITC experiments. ITC experiment parameters include a series of 30 titrations performed at 25°C with a stir speed of 1000 rpm. ITC binding curves were best-fit to one set of sites binding model. Appropriate corrections were applied to eliminate the contribution from the heats of dilution. The K_d values are reported as K_d(apparent) to account for inaccuracy associated with fitting non-ideal/non-sigmoidal isothermograms.

Intrinsic fluorescence measurements and 8-Anilino-1-naphthalenesulfonic acid (ANS) binding:

Intrinsic fluorescence experiments and ANS binding assays were performed at 25°C on a Hitachi F-2500 fluorimeter. All fluorescence measurements were made using a slit width set

to 2.5 nm. Protein samples were loaded into a quartz cuvette of 1 cm optical path length. A protein concentration of 0.1 $\mu\text{g}/\mu\text{L}$ [in 10 mM phosphate buffer containing 100 mM NaCl, and 25 mM $(\text{NH}_4)_2\text{SO}_4$ (pH 7.2)] was used in all intrinsic fluorescence experiments. For samples containing heparin, the glycosaminoglycan was present in 10-times molar excess of the protein concentration to achieve saturated binding of heparin. All intrinsic fluorescence measurements were acquired using an excitation wavelength of 280 nm and an emission wavelength range of 300 nm – 450 nm. ANS binding assays were carried out at protein concentrations of 0.25 $\mu\text{g}/\mu\text{L}$ in 10 mM phosphate buffer containing 100 mM NaCl and 25 mM $(\text{NH}_4)_2\text{SO}_4$ (pH 7.2). ANS binding measurements were performed by adding 1 μL of ANS (from a stock solution of 0.01 μM) into the protein solution such that the concentration of the fluorescent dye increased in 20 μM increments. After each titration, the reaction mixture was excited at 380 nm and the relative fluorescence intensity was measured at 500 nm.

Circular Dichroism Spectroscopy:

Far-UV CD measurements were performed using a protein concentration of 0.5 $\mu\text{g}/\mu\text{L}$ at 25 °C on a Jasco 1500 spectropolarimeter. Protein samples were prepared in 10mM phosphate buffer containing 10 mM NaCl and 25 mM $(\text{NH}_4)_2\text{SO}_4$ (pH 7.2). Protein samples were loaded into a sandwich cuvette with an optical path length of 0.1 cm. Far-UV CD spectra acquired in the presence of heparin, were performed with a protein to heparin ratio of 1:10 to ensure complete heparin saturation of hFGF1. All data were normalized using necessary background corrections and smoothed using the Savitzky-Golay algorithm. In samples containing heparin, the contribution of excess heparin to the observed ellipticity was normalized by making appropriate background corrections.

Limited Trypsin Digestion:

For limited trypsin digestion (LTD) experiments, a 1:100 ratio of enzyme (5 μg) to substrate (500 μg) was dissolved in 10 mM phosphate buffer containing 100 mM NaCl and 25 mM $(\text{NH}_4)_2\text{SO}_4$ at pH 7.2. LTD experiments performed in the presence of heparin contained the glycosaminoglycan in ten-times molar excess of the protein concentration to achieve complete saturation of the protein. hFGF1 samples, incubated at 37°C, were withdrawn at 5-minute intervals and the reaction was stopped with the addition of 100% trichloroacetic acid prior to analysis on SDS PAGE. The intensity of the ~17 kDa band corresponding to hFGF1, as measured by densitometry [using UN-SCAN IT (Silk Scientific Inc., USA)], at time $t = 0$ represents 100% undigested protein. The percentage of protein digested/undigested, at any given time point, was calculated from the ratio of the intensity of the ~17 kDa hFGF1 band at time (t) to the band intensity of the corresponding band at time ($t=0$). In this context, the band intensity of the ~17 kDa band, corresponding to hFGF1, at $t=0$ serves as an internal standard to calculate the percentage of protein (hFGF-1) at any time point (t).

Equilibrium unfolding of hFGF1 mutants:

Thermal and chemical denaturation experiments were completed using a Jasco-1500 spectropolarimeter equipped with fluorescence detector. For thermal unfolding, protein samples were prepared using a protein concentration of 0.5 $\mu\text{g}/\mu\text{L}$ in 10 mM phosphate buffer containing 100 mM NaCl and 25mM $(\text{NH}_4)_2\text{SO}_4$ at pH 7.2. For spectra collected in

the presence of heparin, a protein to heparin ratio of 1:10 was used. Spectra were collected in 5 degrees increments from 20°C to 80°C. The fraction unfolded (f_u) of the protein at each temperature was determined as, $f_u = (R_x - R_{20}) / (R_{80} - R_{20})$ wherein, R_{20} , R_x , and R_{80} are the values of the 308 nm / 350 nm fluorescence ratio at the initial (20°C) temperature, each successive temperature, and the final temperature (80°C), respectively. Each set of data was fit using excel graphing tools. T_m (app), temperature at which 50% of the protein molecules exist in the denatured state(s), was calculated from the plot of fraction of unfolded protein population *versus* temperature as the temperature wherein 50% of the protein population exists in the denatured state(s). As the temperature-induced denaturation of hFGF1 is not completely reversible, the T_m values are reported as T_m (apparent).

Urea-induced equilibrium unfolding of hFGF1 samples was measured using the fluorescence mode on the Jasco – 1500 spectropolarimeter. Urea-induced unfolding experiments were performed at a protein concentration of 0.5 µg/µL protein in 10 mM phosphate buffer containing, 100 mM NaCl and 25mM (NH₄)₂SO₄ at pH 7.2. Far-UV CD and intrinsic fluorescence spectra were recorded individually on a protein sample at increasing concentrations of urea. At each concentration of urea both the molar ellipticity value at 228 nm and the ratio of the 308 nm /350 nm emission intensity was measured. The fraction unfolded of the proteins species, at each concentration of urea, was calculated from both sets of data. Spectra were collected as an average of 3 scans using a quartz cell of 1 cm pathlength.

Nuclear Magnetic Spectroscopy:

NMR spectra were obtained on a Bruker 500 MHz NMR, equipped with a cryoprobe, using $2K \times 256$ data points. ¹H-¹⁵N Heteronuclear single quantum coherence (HSQC) experiments were performed at 25°C using a protein concentration of at least 300 µM in 10 mM phosphate buffer containing 100 mM NaCl and 25 mM (NH₄)₂SO₄ (pH 7.2). Protein samples were isotope-enriched with ¹⁵NH₄Cl using well-established protocols [43]. 2D NMR data were analyzed using Sparky 3.114 software [44]. Composite ¹H-¹⁵N chemical shift perturbation for each residue was calculated using the equation, $[(2 \delta_{NH})^2 + (\delta_N)^2]$. ¹H-¹⁵N chemical shift perturbations, in the presence of heparin, were carefully tracked by acquiring a series of ¹H-¹⁵N HSQC spectra at various heparin: protein ratios. However, for a few residues, the ¹H-¹⁵N chemical shift perturbation(s) could not be precisely monitored and these residues were not considered in the ¹H-¹⁵N chemical shift perturbation plot(s).

Cell proliferation activity:

NIH 3T3 fibroblast cells (supplied by ATCC (Manassas, VA) were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. When cells reached 80-90% confluency, they were incubated overnight in serum-free media at 37°C with 5% CO₂. Cell surface heparin was removed by treating the NIH 3T3 cells with 6 units of heparinase per 10,000 cells for 1 hour at 37°C. Cells were then washed twice with PBS and returned to complete DMEM. Following heparinase treatment, an optimum heparin: protein ratio was determined by conducting the cell proliferation assays at different heparin concentrations and a fixed wt-hFGF1 concentration of 1×10^{-5} µg/µL (10 ng/mL) (Fig. S8). Maximum cell proliferation activity was achieved at a protein: heparin ratio of 1:10, and this ratio was used

in all experiments wherein exogenous heparin was added. In brief, starved 3T3 fibroblasts were distributed in a 96-well plate at a density of 10,000 cells/well. wt-hFGF1 and mutants were individually added at concentrations of 0, 0.4, 2, 10 and 50 ng /mL and incubated for 24 hours. The CellTiter-Glo (Promega, Madison, WI) cell proliferation assay was used following the manufacturer instructions to quantify the proliferation of NIH 3T3 cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Abbreviations:

hFGF1	human acidic fibroblast growth factor-1
wt-hFGF1	wildtype hFGF1
SDM	site directed mutagenesis
CD	circular dichroism
HSQC	heteronuclear single quantum coherence
ATCC	American Type Culture Collection
VMD	Visual Molecular Dynamics
ITC	isothermal titration calorimetry

References

1. Mason IJ, The Ins and Outs of Fibroblast growth factors. 1994, Cell. p. 547–552.
2. Wilkie AOM, Functions of fibroblast growth factors and their receptors. Cell Press, 1995 5(5): p. 500–507.
3. Itoh N, Fibroblast growth factors: from molecular evolution to roles in development, metabolism and disease. J. Biochem, 2011 149(121–130.).
4. Ornitz DM and Nobuyuki I, The Fibroblast Growth Factor Signaling Pathway. WIREs Dev Biol, 2015 4: p. 215–266.
5. Katoh M, Therapeutics Targeting FGF Signaling Network in Human Diseases. Trends in Pharmacological Science, 2016 37(12): p. 1081–1096.
6. Eswarakumar VS, Lax., Cellular Signaling by Fibroblast Growth Factor Receptors Cytokine & Growth Factor Reviews 2005 16: p. 139–149. [PubMed: 15863030]
7. Carter EP, Fearon AE, and Grose RP, Careless talk costs lives: fibroblast growth factor receptor signalling and the consequences of pathway malfunction. Trends Cell Biol, 2015 25(4): p. 221–233. [PubMed: 25467007]
8. Miller D, Compensation by Fibroblast Growth Factor 1 (FGF1) Does Not Account for the Mild Phenotypic Defects Observed in FGF2 Null Mice American Society for microbiology, 2000 20: p. 2260–2268. [PubMed: 10688672]

9. Beenken A, Plasticity in Interactions of Fibroblast Growth Factor 1 (FGF1) N Terminus with FGF Receptors Underlies Promiscuity of FGF1. *J. Biol. Chem.*, 2012 287(5): p.30676–3078.
10. Zakrzewska M, Design of fully active FGF-1 variants with increased stability. *Protein Engineering, Design & Selection*, 2004 17(8): p. 603–611.
11. Dubey VK, et al., Spackling the Crack: Stabilizing Human Fibroblast Growth Factor-1 by Targeting the N and C terminus β -Strand Interactions. *J. Mol. Biol.*, 2007 371: p. 256–268. [PubMed: 17570396]
12. Culajay JF, et al., Thermodynamic characterization of mutants of human fibroblast growth factor 1 with an increased physiological half-life. *Biochemistry*, 2000 39(24): p.7153–8. [PubMed: 10852713]
13. Yu-Peng H and et al., Divergent Synthesis of 48 Heparan Sulfate-Based Disaccharides and Probing the Specific Sugar–Fibroblast Growth Factor - 1 Interaction 2012, *Journal of the American Chemical Society*. p. 20722–20727. [PubMed: 23240683]
14. Brown A, Cooperative Heparin-Mediated Oligomerization of Fibroblast Growth Factor-1 (FGF1) Precedes Recruitment of FGFR2 to Ternary Complexes *Biophysical Journal* 2013 104: p. 1720–1730. [PubMed: 23601319]
15. Hook M, Kjelle ´n L, and Johansson S, Cell-surface glycosaminoglycans *Annu. Rev. Biochem* 1984 53: p. 847–869.
16. Prudovsky I, et al., Folding of Fibroblast Growth Factor 1 Is Critical for Its Nonclassical Release. *Biochemistry*, 2016 55(7): p. 1159–67. [PubMed: 26836284]
17. Ornitz DM and Itoh N, Fibroblast growth factors. *Genome Biology*, 2001 2(3): p. 1–12.
18. Harmer N, Insights into the role of heparan sulphate in fibroblast growth factor signalling. *Biochemical Society Transactions*, 2006 34(3): p. 442–445. [PubMed: 16709182]
19. Pellegrini L, et al., Crystal structure of fibroblast growth factor receptor ectodomain bound to ligand and heparin. *Nature*, 2000 407(6807): p. 1029–1034. [PubMed: 11069186]
20. Pellegrini L, Role of heparan sulfate in fibroblast growth factor signalling: a structural view. *Current Opinion in Structural Biology* 2001 11: p. 629–634. [PubMed: 11785766]
21. DePaz and JoseÁ-Luis, The Activation of Fibroblast Growth Factors by Heparin: Synthesis, Structure, and Biological Activity of Heparin-Like Oligosaccharides *ChemBiochem*, 2001 2: p. 673–685. [PubMed: 11828504]
22. Szlachcic A, et al., Structure of a highly stable mutant of human fibroblast growth factor 1. *Acta Crystallographica Section D-Biological Crystallography*, 2009 65: p. 67–73.
23. Arunkumar AI, Oligomerization of acidic fibroblast growth factor is not a prerequisite for its cell proliferation activity. *Protein Science* 2002 11: p. 1050–1061. [PubMed: 11967362]
24. Erzurum VZ, et al., R136K fibroblast growth factor-1 mutant induces heparin-independent migration of endothelial cells through fibrin glue. *Journal of Vascular Surgery*, 2003 37(5): p. 1075–1081. [PubMed: 12756357]
25. Duarte M, Thrombin induces rapid PAR1-mediated non-classical FGF1 release. *Biochemical and Biophysical Research Communications*, 2006 350: p. 604–609. [PubMed: 17027650]
26. Thallapuranam SK, Engineered Compositions of FGF and Methods of Use Thereof, *Law AIP*, Editor. 2016: United States.
27. DiGabriele A, Structure of a heparin-linked biologically active dimer of fibroblast growth factor. *Nature*, 1998 393: p. 812–817. [PubMed: 9655399]
28. Canales A, et al., Solution NMR structure of a human FGF-1 monomer, activated by a hexasaccharide heparin-analogue. *Febs Journal*, 2006 273(20): p. 4716–4727. [PubMed: 16995857]
29. Callis P, Binding phenomena and fluorescence quenching. II: Photophysics of aromatic residues and dependence of fluorescence spectra on protein conformation *Journal of Molecular Structure*, 2014 1077: p. 22–29.
30. Gabellieri E and Strambini GB, Perturbation of Protein Tertiary Structure in Frozen Solutions Revealed by 1-Anilio-8-Napthalene Sulfonate Fluorescence. *Biophysical Journal*, 2003 85(5): p. 3214–3220. [PubMed: 14581221]

31. Lindh E, Increased Resistance of Immunoglobulin a Dimers to Proteolytic Degradation after Binding of Secretory Component. *J Immunol*, 1975 114: p. 284–286. [PubMed: 1090649]
32. Zakrzewska M, et al., Highly stable mutants of human fibroblast growth factor-1 exhibit prolonged biological action. *J Mol Biol*, 2005 352(4): p. 860–75. [PubMed: 16126225]
33. Zakrzewska M, et al., Increased Protein Stability of FGF1 Can Compensate for Its Reduced Affinity for Heparin. *Journal of Biological Chemistry*, 2009 284(37): p. 25388–25403. [PubMed: 19574212]
34. Xia X, et al., Properties of 2nd-Generation Fibroblast Growth Factor-1 Mutants for Therapeutic Application. *PLOS-One*, 2012 7(11): p. e48210. [PubMed: 23133616]
35. Wong P, Analysis of Putative Heparin-binding Domains of Fibroblast Growth Factor-1. *J. Biol. Chem*, 1995 270(43): p. 25805–25811. [PubMed: 7592764]
36. Burnett MJ, Somasundaram T, and Blaber M, An atomic resolution structure for human fibroblast growth factor 1. *Proteins-Structure Function and Bioinformatics*, 2004 57(3): p. 626–634.
37. Olsson, et al., Propka3: consistent treatment of internal and surface residues in empirical pKa predictions. *J. Chem. Theory Comput*, 2011 7: p. 525–537. [PubMed: 26596171]
38. Best, et al., Optimization of the additive CHARMM all-atom protein force field targeting improved sampling of the backbone ϕ , ψ and side-chain χ_1 and χ_2 dihedral angles. *J. Chem. Theory Comput*, 2012 8: p. 3257–3273. [PubMed: 23341755]
39. Martyna GJ, Tobias DJ, and Klein ML, Constant pressure molecular dynamics algorithms. *J. Chem. Phys*, 1994 101: p. 4177–4189.
40. Humphrey W, Dalke A, and Schulten K, VMD: Visual Molecular Dynamics. *J. Mol. Graph*, 1996 14: p. 27–28, 33–38.
41. Froger A and Hall JE, Transformation of Plasmid DNA into E. coli Using the Heat Shock Method. *J. Visualized Experiments*, 2007 6: p. 253.
42. Eberle Davis J, et al., Effect of Extension of the Heparan Sulfate Binding Pocket on the Structure, Stability, and Cell Proliferation Activity of the Human Acidic Fibroblast Growth Factor. *Biochemistry and Biophysics Reports*, 2018 13: p. 45–57. [PubMed: 29556563]
43. Marley J, Lu M, and Bracken C, A method for efficient isotopic labeling of recombinant proteins. *J. of Biomolecular NMR*, 2001 20: p. 71–75.
44. Goddard TK, DG, SPARKY 3. University of California, San Francisco.

Highlights:

- Mutation of residues P135 and R136 alters heparin-binding affinity of hFGF1.
- The cell proliferation activity of hFGF1 is not correlated to its heparin-binding affinity.
- Mutation R136E increases hFGF1 thermal stability and activity independent of heparin.

Figure 1:

The spatial orientation of P135 (highlighted in pink) in the loop between beta strands XI and XII in relation to heparin (blue sticks) and charged residues K119, R133, and R136, of which, R133 and R136 are critically involved in heparin binding. The C α atom of P135 is positioned 3.4 Å and 6.2 Å from the functional side chains of R133 and R136 respectively. It is 5.4 Å from the side chain e-amino group of K119 (PDB 2ERM) [28].

Figure 2:

Far-UV CD spectra of wt-hFGF1 and all hFGF1 mutants in the absence (Panel-A) and presence (Panel-B) of heparin reveals that beta-barrel structure is not significantly compromised by any of the designed mutations. Wt-hFGF1 (Δ), P135E ($\Diamond$), P135K ($\bigcirc$), P135Q ($\square$), P135E/R136E ($-$), P135K/R136E ($\times$). Far-UV CD measurements were performed using a protein concentration of $\sim 0.5 \mu\text{g}/\mu\text{L}$ at 25°C on a Jasco 1500 spectropolarimeter. Protein samples were prepared in 10 mM phosphate buffer containing 10 mM NaCl and 25 mM $(\text{NH}_4)_2\text{SO}_4$ (pH 7.2).

Figure 3:

Intrinsic fluorescence spectra for wt-hFGF1 and the hFGF1 mutants in the absence (Panel-A) and presence (Panel-B) of heparin. wt-hFGF1 (Δ), P135E ($\diamond$), P135K ($\circ$), P135Q ($\square$), P135E/R136E ($-$), P135K/R136E ($\times$). Insert figure in Panel-A depicts the fluorescence spectra of the native (N) and 8 M urea denatured (D) state(s) wt-hFGF1. All intrinsic fluorescence spectra were obtained at a protein concentration of 0.1 $\mu\text{g}/\mu\text{L}$ [in 10 mM phosphate buffer containing 100 mM NaCl, and 25 mM $(\text{NH}_4)_2\text{SO}_4$ (pH 7.2)].

Figure 4:

ANS binding curves for wt-hFGF1 and the hFGF1 mutants in the absence (Panel-A) and presence (Panel-B) of heparin. wt-hFGF1 (Δ), P135E ($\diamond$), P135K ($\circ$), P135Q ($\square$), P135E/R136E (-), P135K/R136E (X). ANS binding assays were carried out with protein concentrations of 0.25 $\mu\text{g}/\mu\text{L}$ in 10 mM phosphate buffer containing 100 mM NaCl and 25 mM $(\text{NH}_4)_2\text{SO}_4$ (pH 7.2).

Figure 5:

LTD of wt-hFGF1 and all hFGF1 mutants in the absence (Panel-A) and presence (Panel-B) of heparin, wt-hFGF1 (Δ), P135E (◇), P135K (○), P135Q (□), P135E/R136E (-), P135K/R136E (X). Error bars represent deviation between a pair of trypsin digestion experiments. For limited trypsin digestion (LTD) experiments, a 1:100 ratio of enzyme (5 μg) to substrate (500 μg) was dissolved in 10 mM phosphate buffer containing 100 mM NaCl and 25 mM (NH₄)₂SO₄ at pH 7.2.

Figure 6:

Panel – **A**, The ^1H - ^{15}N HSQC of wtFGF (red) is superimposed onto P135K (blue). Panel – **B**, the chemical shift perturbation plot of the P135K mutant. The dashed line signifies an arbitrary threshold above which ^1H - ^{15}N chemical shift perturbations are considered significant. The ^1H - ^{15}N chemical shift perturbation of individual residues were calculated using the formula, $([(2 \delta_{NH})^2 + (\delta_N)^2])$. A minimum protein concentration of 300 μM in 10 mM phosphate buffer containing 100 mM NaCl and 25 mM $(\text{NH}_4)_2\text{SO}_4$ (pH 7.2) was used.

Figure 7:

Panel – **A**, The HSQC of wtFGF (red) is superimposed onto P135E (blue). Panel – **B**, the chemical shift perturbation from the P135E mutant. The dashed line signifies an arbitrary threshold above which ^1H – ^{15}N chemical shift perturbations are considered significant. The ^1H – ^{15}N chemical shift perturbation of individual residues were calculated using the formula, $([(2 \delta_{NH})^2 + (\delta_N)^2])^{1/2}$. A minimum protein concentration of 300 μM in 10 mM phosphate buffer containing 100 mM NaCl and 25 mM $(\text{NH}_4)_2\text{SO}_4$ (pH 7.2) was used.

Figure 8:

Thermal (Panel-A) and urea-induced (Panel-B) equilibrium unfolding of wt-hFGF1 and hFGF1 mutants in the presence and absence of heparin. Protein unfolding was monitored by changes in intrinsic fluorescence and the fraction of unfolded protein was calculated from changes in fluorescence intensity ratio at 308 nm/350 nm. Unfolding profile in the absence of heparin: wt-hFGF1 (Δ), P135E ($\diamond$), P135K ($\circ$), P135Q ($\square$), P135E/R136E ($-$), P135K/R136E ($*$). Unfolding profile in the presence of heparin: wt-hFGF1 ($\blacktriangle$), P135E ($\blacklozenge$), P135K ($\bullet$), P135Q ($\blacksquare$), P135E/R136E ($\times$), P135K/R136E ($+$). For thermal unfolding experiments, the concentration of protein used was 0.5 $\mu\text{g}/\mu\text{L}$ in 10 mM phosphate buffer containing 100 mM NaCl and 25mM $(\text{NH}_4)_2\text{SO}_4$ at pH 7.2. Urea-induced unfolding experiments were performed at a protein concentration of 0.5 $\mu\text{g}/\mu\text{L}$ protein in 10 mM phosphate buffer containing, 100 mM NaCl and 25mM $(\text{NH}_4)_2\text{SO}_4$ at pH 7.2.

Figure 9:

Isothermograms representing the titration of wt-hFGF1 and the individual designed mutants of hFGF1 with heparin. The top panel displays the raw heat changes from heparin-protein interaction and the bottom panel displays the best-fit of the binding curve using a one set-of-sites binding model [14]. All data have been corrected for heats of dilution. Protein and heparin samples were in 10 mM phosphate buffer with 100 mM NaCl and 25 mM (NH₄)₂SO₄ (pH 7.2) and were degassed prior to titration. A protein concentration of 50 μM and a heparin concentration of 500 μM was consistently used to maintain a 1:10 ratio of protein to heparin in all ITC experiments. The endothermic phase in the isothermogram representing the P135E versus heparin titration appears to stem from the minor imbalance in the concentration of the buffer components in the titer and the titrant.

Figure 10:

Proliferation of heparinase-treated NIH 3T3 cells by wt-hFGF1 and the designed hFGF1 mutants in the absence (Panel-A) and in the presence (Panel-B) of exogenous heparin. Standard errors were determined from triplicate experiments.

Table-1:

Thermal stability of wt-hFGF1 and its designed mutants

	T_m °C		T_m	C_m (M)		C_m
	<u>- heparin</u>	<u>+ heparin</u>		<u>- heparin</u>	<u>+ heparin</u>	
wt-hFGF1	48 ± 0.72	69 ± 0.68	21	1.8 ± 0.11	4.2 ± 0.09	2.4
P135E	43 ± 1.3	59 ± 0.43	16	1.5 ± 0.32	4.1 ± 0.17	2.6
P135K	42 ± 1.0	63 ± 0.81	21	1.4 ± 0.27	3.1 ± 0.28	1.7
P135Q	44 ± 0.40	60 ± 0.50	16	1.4 ± 0.27	2.9 ± 0.15	1.5
P135E/R136E	53 ± 0.87	58 ± 0.46	5	1.8 ± 0.67	2.3 ± 0.60	0.5
P135K/R136E	48 ± 0.91	48 ± 0.55	0	1.8 ± 0.26	2.7 ± 0.32	0.9