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Effect of extension of the heparin binding pocket on the structure, stability, and cell proliferation activity of the human acidic fibroblast growth factor

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ABSTRACT

Acidic human fibroblast growth factor (hFGF1) plays a key role in cell growth and proliferation. Activation of the cell surface FGF receptor is believed to involve the glycosaminoglycan, heparin. However, the exact role of heparin is a subject of considerable debate. In this context, in this study, the correlation between heparin binding affinity and cell proliferation activity of hFGF1 is examined by extending the heparin binding pocket through selective engineering via charge reversal mutations (D82R, D84R and D82R/D84R). Results of biophysical experiments such as intrinsic tryptophan fluorescence and far UV circular dichroism spectroscopy suggest that the gross native structure of hFGF1 is not significantly perturbed by the engineered mutations. However, results of limited trypsin digestion and ANS binding experiments show that the backbone structure of the D82R variant is more flexible than that of the wild type hFGF1. Results of the temperature and urea-induced equilibrium unfolding experiments suggest that the stability of the charge-reversal mutations increases in the presence of heparin. Isothermal titration calorimetry (ITC) data reveal that the heparin binding affinity is significantly increased when the charge on D82 is reversed but not when the negative charge is reversed at both positions D82 and D84 (D82R/D84R). However, despite the increased affinity of D82R for heparin, the cell proliferation activity of the D82R variant is observed to be reduced compared to the wild type hFGF1. The results of this study clearly demonstrate that heparin binding affinity of hFGF1 is not strongly correlated to its cell proliferation activity.

1. Introduction

FGFs are a family of polypeptides involved in a wide range of core signaling processes that govern cell growth, cell proliferation, cell differentiation, stress response and wound healing [1–7]. FGFs exert their action(s) by binding to their cell surface receptors (FGFRs) [1,6–9]. FGF1 is the only member of the FGF family that binds with high affinity to all four types of FGFRs [10,11]. Formation of the hFGF1/FGFR complex, initiates the processes of dimerization and autophosphorylation of the intracellular tyrosine kinase domain, ultimately triggering downstream signaling [12].

hFGF1-receptor interaction is believed to be highly dependent on heparin, a glycosaminoglycan that is commonly found in the extracellular matrix of mammalian cells at concentrations up to 10^6 units per cell [5,13–15]. Heparin consists of long, unbranched, helical chains of repeating disaccharide units, which are heavily sulfonated [13]. Heparin is also believed to be critical for the biological activity of hFGF1 [16]. Crystal structures of the hFGF1/FGFR complex suggest that heparin directly contacts both hFGF1 and the receptor [16].

Crystal and solution structures of the heparin-FGF complex show that the glycosaminoglycan binds to a cluster of positively charges residues, which are located at the c-terminal end of the molecule. The residues involved in the putative heparin-binding pocket of hFGF1 include K126, K127, K132, R133, R136, and K142 [17]. These heparin-binding amino acids form a region of concentrated positive charge that renders hFGF1, in its unbound form, to be relatively unstable and prone to thermal and proteolytic degradation [4,18–20]. Therefore, hFGF1 binding to heparin increases the stability of the growth factor [8,9].

Abbreviations: hFGF1, human fibroblast growth factor-1; 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

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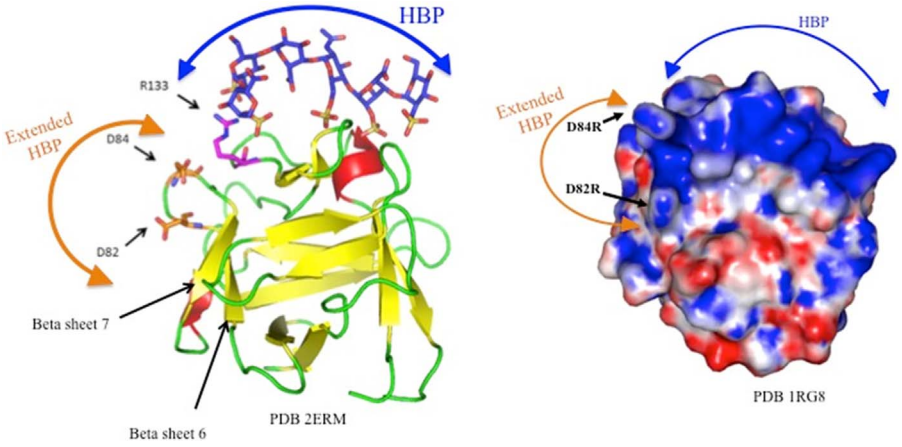

Fig. 1. Left -Three-dimensional structure of hFGF1 (PDB 2ERM) depicting the side-chain of the positively charged residue R133 in the heparin-binding pocket. The heparin-binding pocket has been extended through reversal of charge on D82 (D82R) and D84 (D84R). Right - Representation of the electrostatic potential in the three-dimensional structure of hFGF1 showing that charge reversal(s) at D82 and D84 extends the heparin-binding region of hFGF1 (PDB 1RG8).

Furthermore, heparin's interaction with FGFRs stabilizes the hFGF1-FGFR binary complex [5,16,21,22]. However, there has been a long-standing debate on whether heparin is obligatory for hFGF1 activity. The initial “dogma” describes heparin's role as a mandatory co-receptor that is critical for cell signaling events triggered by hFGF1 [14,16,23,24]. However, there are significant reports to the contrary, which suggest that heparin is only necessary for stabilizing the growth factor [4,12,18,20,25,26]. In this context, the debate on the exact role of heparin in the FGF1 signaling process is still an open question.

Several studies using a site-directed mutagenesis approach have identified several conserved residues, which, when appropriately mutated, increased the thermodynamic stability of hFGF1. In addition, several mutant forms of hFGF1 have exhibited mitogenic activity even in the absence of heparin [4,12,18–20,25,26]. One notable mutation is the charge-reversed substitution K132E in the heparin-binding pocket, which was found to reduce hFGF1's affinity for heparin [12]. Interestingly, it has been shown that introduction of thermally stabilizing mutations into hFGF1 variants with reduced affinity for heparin, such as K132E, have been shown to compensate for lack of heparin involvement in FGFR activation [12]. While these studies suggest that heparin binding to hFGF1 is not critical for the protein's mitogenic activity, heparin is still opined to have a critical role in hFGF1 receptor binding and signaling [9,27–29].

One approach to assess the role of heparin that has yet to be investigated is the evaluation of hFGF1 activity using mutations that are predicted to increase heparin binding. In this context, we have studied the correlation between heparin-binding affinity and cell proliferation activity of hFGF1 by designing charge-reversal mutations in the vicinity of the heparin binding pocket involving the substitution of aspartic acid for arginine at position 82 and 84 (PDB 2ERM) [17]. Specifically, D82R and D84R mutations have been designed to extend the heparin-binding interface on hFGF1. Crystal structures of hFGF1, both with and without heparin, were used to measure the distance between the carboxyl side chain group of D82 and the guanidinium side chain group of R133. Comparison of these measurements reveals that the position of D82 in the heparin-bound hFGF1 structure is 6 A° farther away from the heparin-binding region than it is in the native hFGF1 structure. D82N is also included as a control mutant to determine if a lack of negative charge at this position influences the protein's structure, stability, activity, and interaction differently than the D82R mutant.

Results of this study show that charge reversal at position 82 destabilizes hFGF1 structure. Furthermore, although charge reversals D82R and D84R increase heparin-binding affinity, they do not concomitantly increase the cell proliferation activity of hFGF1. The results obtained in this study strongly suggest that heparin merely increases the bioavailability of hFGF1 at the cell surface and thereby enhances the probability of a productive hFGF1-receptor interaction. For this study, all protein samples were derived from expression in *E. coli*, an expression system that lacks post-translational modification. However, hFGF1 is not known or predicted to undergo post-translational modification that would potentially affect the heparin binding.

2. Results and discussion

Human acidic fibroblast growth factor (hFGF1) is a beta-barrel protein with 12 antiparallel beta-strands. The canonical heparin-binding pocket is located at the c-terminal end between beta strands 10 and 12. Positively charged residues densely populate the heparin-binding pocket in the protein. Site-directed mutagenesis studies have shown that the positively charged residues in the heparin-binding pocket are critical for the heparin binding affinity of hFGF1 [30,31]. In this study, we have used low weight molecular heparin as it is well known that high molecular weight heparin is polydispersed, containing a heterogeneous mixture of heparin molecules of varying chain lengths. The high polydispersity poses significant technical challenge in the interpretation of the NMR results. In addition, in our experience, the high polydispersity also presented serious problems in calculating the average molecular weight, which in-turn adversely influenced the heparin concentration determination(s). The low polydispersity of the low molecular heparin significantly diminished the above-mentioned technical difficulties. Furthermore, the low molecular weight (M_r~ 3000 Da) heparin used has been estimated to be 8–12 units long and multiple studies have shown that heparin with a chain length of 8-units has been shown to be sufficient to facilitate optimal FGF-induced cell signaling [13,32,33].

2.1. Spatial proximity of D82/D84 to the putative heparin-binding pocket

Residues D82 and D84 are located on the protein surface within the linker region connecting beta strands 6 and 7 (Fig. 1). In the folded conformation of hFGF1, both D82 and D84 residues appear to be fully solvent-exposed [17]. Thus, neither amino acid is involved in the inner core network of hydrogen bonding or electrostatic interactions stabilizing the three-dimensional structure of the protein. The side chain carboxyl groups of D82 and D84 are located within a spatial distance of 5 A° from T83, L86, L87, and Y88. Neither D82 nor D84 is a part of the canonical heparin-binding pocket. The position of both D82 and D84 in relation to R133, which is in the midst of the heparin-binding pocket, was measured using crystal structures of native hFGF1 in the presence (PDB 2ERM) and absence of heparin (PDB 1RG8) [17,32]. In the absence of heparin, D82 and D84 are positioned ~6.9 Å and ~6.1 Å, respectively, away from the critical heparin-binding residue R133 (PDB 1RG8) [32]. In the presence of a hexasaccharide heparin chain (PDB 2ERM), D84 is shifted modestly closer to R133 (~5.8 Å) while D82 is measured at ~12.8 Å from R133.

Using the crystal structure of hFGF1-heparin-FGFR ternary complex