FGFs, heparan sulfate and FGFRs: complex interactions essential for development

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Summary

Fibroblast growth factors (FGFs) comprise a large family of developmental and physiological signaling molecules. All FGFs have a high affinity for the glycosaminoglycan heparin and for cell surface heparan sulfate proteoglycans. A large body of biochemical and cellular evidence points to a direct role for heparin/heparan sulfate in the formation of an active FGF/FGF receptor signaling complex. However, until recently there has been no direct demonstration that heparan is required for the biological activity of FGF in a developmental system in vivo. A recent paper by Lin et al.[1] has broken through this barrier to demonstrate that heparan sulfate is essential for FGF function during *Drosophila* development. The establishment of a role for heparan sulfate in FGFR activation in vivo suggests that tissue-specific differences in the structure of heparan may modulate the activity of FGF. *BioEssays* 22:108–112, 2000.

Introduction

Fibroblast growth factor (FGF) was originally purified from bovine pituitary gland as a mitogen that could stimulate the growth of NIH3T3 cells. The discovery that FGF has a high affinity for heparin facilitated its purification and led to the observation that heparin could stabilize FGF from heat and proteolysis. Over the last 25 years nineteen *Fgfs* and four *Fgf receptors (Fgfrs)* have been identified in vertebrate species, two *Fgfrs* and one *Fgf* have been identified in *Drosophila* (*Breathless, btl; Heartless, htl; branchless, bnl*) and one *Fgf/Fgfr* pair has been identified in *C. elegans (Egl-17/Egl-15)*. The biological activities of FGFs have been studied both in vitro and in vivo and include roles for FGFs in regulating cell growth, survival, differentiation and migration. Studies addressing the biochemical activities of FGFs have focused on the specificity of interactions between FGFs and FGF receptors (FGFRs), on factors that affect the stability of FGFs and on the composition and mechanism of the active FGF/FGFR signaling complex. The identification of heparin/heparan sulfate (HS) as an active and essential component of the FGF/FGFR signaling complex suggested that FGF activity and specificity can be modulated by HS and in turn by enzymes that synthesize and degrade HS. The findings of Lin et al.[1], that mutations in two enzymes involved in the biosynthesis of heparan result in defects in FGF signaling during development, strongly support this hypothesis. In addition, several other studies examining the phenotypes resulting from mutations in genes involved in sulfate activation[2], sulfate transport[3] or heparan 2-O-sulfation[4] suggest an etiology that is linked to FGF signaling pathways. FGFs are essential developmental signaling molecules.

A large body of evidence shows that FGF pathways are required for vertebrate and invertebrate development (reviewed in Refs. 5, 6). The roles of some of the FGFs and FGFRs in development have been addressed by targeted gene disruption in the mouse, by the identification of mutations in *Drosophila* and *C. elegans* and by over-expression in chicken and *Xenopus* embryos. These studies demonstrate that FGFs often signal directionally and reciprocally across epithelial-mesenchymal boundaries and have the ability to organize or pattern tissue surrounding the source of FGF.[7,8] The integrity of these signaling pathways requires extremely tight regulation of FGF activity and receptor specificity. For example, in vertebrate limb development, mesenchymally expressed FGF10 induces the formation of the overlying apical ectodermal ridge which subsequently expresses *Fgf8,* a molecule which signals back to the underlying mesoderm. This directional signaling initiates feedback loops and along with other signaling molecules regulates the outgrowth and patterning of the limb. In *Drosophila* and *C. elegans* FGF signaling also regulates the development of both epithelial and mesenchymal lineages and, as in vertebrates, it appears that these signaling pathways are highly specific. Mutations in the *Drosophila Fgfr, Breathless (btl)* and its ligand, *Branchless (bnl)* cause defects in tracheal cell migration and subsequent epithelial branching.[9-11] In contrast, the *Drosophila Fgfr Heartless (htl)* regulates dorsolateral mesoderm migration and the specification of cardiac and muscle cell fates.[12-14] In *C. elegans* the *Fgfr (Egl-15)* and its ligand *(Egl-17)* are essential for the normal migration of sex myoblasts.[15-17]

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Biochemical features of FGFs

Structural considerations

All FGFs share an internal core region of similarity with 28 highly conserved, and six invariant amino acid residues. FGFs range in molecular weight from 17 to 34 kDa in verte-

brates and up to 84 kDa in Drosophila. Structural studies on FGF1 and FGF2 demonstrate that these proteins adopt a β trefoil structure which contains four-stranded β sheets arranged in a triangular array (reviewed in Ref. 18). The loop between β strands 10 and 11 contain several basic amino acid residues which form the primary heparin binding site on FGF2. Regions thought to be involved in receptor binding include the β8-β9 loop and are distinct from the heparin binding site.[19-21]

FGFs have a high affinity for heparin/HS

Interactions between FGF and heparin have been shown to stabilize FGF1 and FGF2 to thermal denaturation and protect FGF2 from proteolysis. The affinity of FGFs for HS proteoglycans severely limits their diffusion and release into interstitial spaces.[22,23] FGFs are therefore likely to exert their effects very close to their site of production or may be released by heparin/HS degrading enzymes as soluble FGF/HS complexes. The binding of FGFs to heparin/HS results in the formation or stabilization of dimers and higher order oligomers along the proteoglycan chain.[24-26] FGFs can also form "trans" dimers with a heparin molecule bound between two FGF molecules.[27] However, it is still controversial whether a heparin/HS-FGF dimer is the essential component of the FGF/FGFR signaling complex (reviewed in Ref. 28). Nevertheless, it has been established that heparin is required for high affinity binding of FGF to the FGFR in cells that are unable to synthesize cell-surface HS, in cells pretreated with heparin degrading enzymes or in cells pretreated with inhibitors of sulfation.[29,30] Furthermore, it is now well established that heparin is required for efficient signal transduction in cells deficient in HS.[31] Additional studies have shown that heparin/HS acts to increase the affinity and half life of the FGF/FGFR complex (reviewed in Refs. 5, 32).

FGFRs

FGFRs are members of the receptor tyrosine kinase superfamily.[33] The extracellular ligand binding region of vertebrate FGFRs contains three immunoglobulin-like (Ig-like) domains, a stretch of acidic amino acids between Ig-like domains I and II, and a heparin binding domain.[33,34] Alternative splicing in Ig domain III dramatically changes the specificity of the FGFR for certain FGFs. This splicing event is tissue-specific and is essential for directional FGF signaling across epithelial-mesenchymal boundaries (such as in the developing limb bud). In contrast, another alternative splicing event, that eliminates Ig domain I, has little affect on ligand binding and at present has no well-defined biological function.[35-37] Binding studies comparing similarly spliced FGFRs demonstrate that multiple regions of the FGFR regulate ligand binding specificity and that one FGFR can interact with two molecules of FGF2.[35] The *Drosophila* BTL and *C. elegans* EGL-15 FGFRs are structurally very similar to vertebrate FGFRs; both contain three extracellular Ig-like domains with an acidic region between Ig-like domains I and II. The *Drosophila* HTL receptor is also similar but has an additional two amino terminal Ig-like domains.

Structural features of the FGFR must account for the binding specificity of a large number of ligands and for a mechanism for activation by FGF and heparin/HS. Interestingly, a heparin/HS binding site consisting of a stretch of 18 conserved residues (K18K) has been identified on the FGFR by digesting the FGFR with proteases in the presence of heparin.[34] This K18K sequence is essential for receptor activity and by itself has the capacity to interact with heparin. The identification of this site suggested a model in which heparin could form a bridge between FGF and the FGFR. Recent crystallization of a fragment of the FGFR with FGF2 identified the location of an FGF binding site in a region of the FGFR that includes Ig-like domain II and III and the intervening linker domain.[19] Interestingly, these studies also identified a potential heparin binding cleft in the FGFR that incorporates the K18K peptide[34] and that is contiguous with the heparin binding site on FGF2. This observation supports a model in which heparin/HS bridges an FGF/FGFR complex.

Is heparin/HS necessary for FGF function in vivo?

A extensive amount of biochemical and biological data suggests that heparin/HS is a part of the FGF/FGFR signaling complex. However, the importance of HS for FGF activity in vivo has remained controversial. Lin et al [1] demonstrate that some of the phenotypes in *sgl* and *sfl* null embryos are the same as that seen in *htl*, *bnl* and *btl* null embryos. Furthermore, epistasis studies demonstrate a genetic interaction between these genes with *sgl* and *sfl* lying upstream of *htl*, *bnl* and *btl*.

Heparin and HS are heterogeneously sulfated linear polymers (M_r 5,000 to 100,000) containing repeating disaccharide subunits of hexuronic acid (iduronic or glucuronic acid) and D-glucosamine.[38] HS proteoglycans are located on the surface of most cells and within the extracellular matrix of most tissues. The biosynthetic pathway for HS (Fig. 1A) includes the sequential addition of sugars to the heparan chain followed by modification of heparan, first by deactylation/N-sulfation, and subsequently by 2-O and 6-O sulfation. In *Drosophila,* the *sugarless* (*sgl*) gene encodes an enzyme which catalyzes the formation of UDP-D-glucuronic acid, an essential building block of heparan.[39] Biochemical and genetic proof that Sgl activity is essential for heparan biosynthesis and for HS activity in vivo comes from experiments in which UDP-glucuronic acid or HS, injected into *sgl* null embryos, rescued the *sgl* phenotype and in which injection of heparinases into wild type embryos phenocopied the *sgl* phenotype.[1] FGF binding and mitogenic assays show

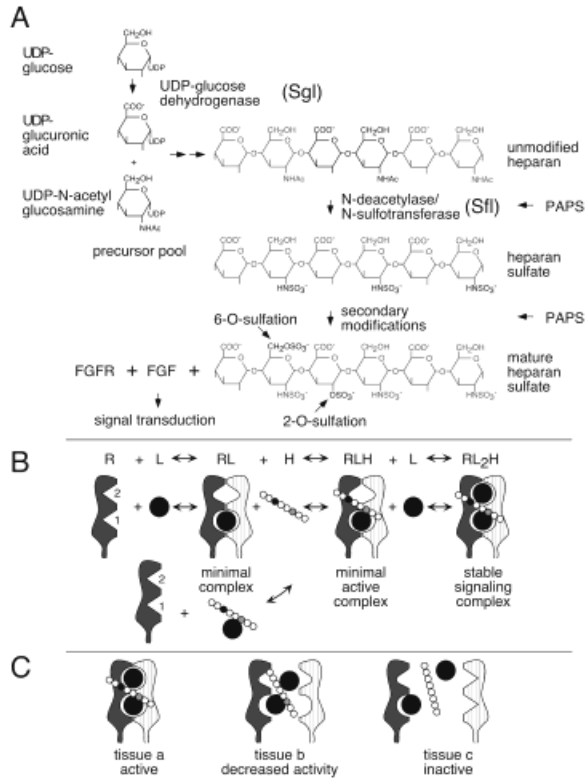

Figure 1. Biosynthetic pathway for heparan sulfate and models for receptor activation. **A**: HS biosynthesis involves the addition of N-acetylglucosamine and glucuronic acid to a proteoglycan core protein. In *Drosophila,* the *sugarless* (*sgl*) gene encodes a homologue of UDP-D-glucose dehydrogenase, which catalyzes the formation of UDP-D-glucuronic acid, an essential building block of heparan.[39] The *sulfateless (sfl)* gene encodes an enzyme with combined N-deacetylase/N-sulfotransferase activity. During and following synthesis the heparan chain is extensively modified by this enzyme. This modification must precede any secondary modifications of heparan. Secondary modifications include C5 epimerization of glucuronic acid to iduronic acid and 2-O- and 6-O-sulfation of the N-sulfated regions of heparan sulfate. 2-O- and N-sulfated heparan can activate FGF and 6-O-sulfated heparan is thought to be necessary for receptor activation and signal transduction. PAPS (phosphoadenosine-phosphosulfate) is the activated substrate for enzymes that modify heparan. Biosynthesis of PAPS requires active transport of sulfate into cells and the enzymes ATP sulfurylase and APS kinase. Mutations anywhere along this pathway can affect the synthesis of heparan sulfate as well as other sulfated molecules. **B**: Model showing the sequential formation of FGF/FGFR signaling complexes of increasing stability and activity. R, FGFR (shaded-first receptor to bind FGF, lightly hatched-second receptor recruited to the initial FGF/FGFR complex); L, FGF ligand (large filled circle); H, heparan sulfate (oooooo) with filled circles indicating 6-O-sulfation and shaded circles indicating 2-O-sulfation; 1, primary FGF binding site; 2, secondary FGF binding site; **C**: Tissue-specific secondary modifications of HS may affect the formation or activity of an FGF/FGFR signaling complex. Tissue a, active signaling complex containing an optimal HS molecule; Tissues b and c, decreased activity of the FGF/FGFR signaling complex resulting from inactive or inhibitory HS molecules.

that highly sulfated regions of heparin are significantly more active than undersulfated regions of heparan and that 2-O-sulfation is required for FGF/FGFR binding but that additional 6-O-sulfation is required for mitogenic activity.[40-42] In *Drosophila*, the *sulfateless* (*sfl*) gene encodes an enzyme which catalyzes the N-deacetylation/N-sulfation of heparan to heparan sulfate. This mutation results in the formation of unmodified heparan chains which cannot activate FGF.[1] Interestingly, mutation of a 2-O-sulfotransferase in mouse results in defects in kidney, bone and eye development, phenotypes which may result from impaired FGF signaling pathways.[4]

The FGF/FGFR signaling complex

Our understanding of the nature of the active signaling complex is still evolving. Current evidence suggests that FGF may form an initial low affinity 1:1 complex with the FGFR which may be stabilized by heparin (Fig. 1B).[19,43,44] This minimal complex may allow transient receptor dimerization and may signal at high ligand concentrations (minimal complex in Fig.1B). In the presence of appropriate HS molecules this complex becomes stabilized and activated.[44] Subsequent binding of a second molecule of FGF may then lead to a more stable 2:2 FGF/FGFR signaling complex.

The formation of a minimal FGF/FGFR complex in the absence of heparin/HS is supported by recent crystallography studies in which an FGF2/FGFR crystal formed in the absence of heparin.[19] Interestingly, examination of the crystal structure identified a potential heparin/HS binding site that bridges FGF2 and the FGFR. In another experiment, cross-linked FGF2/heparin monomers were shown to activate the FGFR in HS deficient cells and FGF7 was shown to form a heparin-dependant 2:1 complex with its receptor.[43,44] These experiments support the concept of a minimal functional complex (Fig. 1B). However, FGF2 binding studies with soluble or cell surface FGFRs in the absence of HS identified two binding sites that interact cooperatively.[35,45]

Interestingly, heparin was found to significantly increase the affinity for the first molecule of FGF.[45] These data suggest that the stable FGF/FGFR signaling complex includes HS and has a ratio of 2 FGFs to 2 FGFRs.

The experiments of Lin et al.[1] examine the requirement for HS to form a stable signaling complex by demonstrating that over expression of *Bnl* can partially rescue the *sfl* and *sgl* phenotype. These experiments demonstrate that in vivo, high levels of FGF can activate the FGFR in the absence of heparan. Collectively, these data suggest that there are multiple steps in the formation of a stable FGF/FGFR signaling complex and support a model in which FGF alone or FGF/HS binds the FGFR and leads to FGFR dimerization and activation (Fig. 1B). The role of HS and possibly a second molecule of ligand would then be to stabilize the active complex for a long enough period of time to elicit a

specific response. Consistent with this, cell culture assays have shown that stimulation of maximal DNA synthesis requires the formation of a stable FGF/FGFR complex over a minimum 12 h period.[46] In contrast, transient activation of the FGFR (assayed by immediate early gene induction) may occur in the absence of heparin.[47] Together, these studies suggest that to fully activate the FGFR a trimolecular complex forms between FGF, the FGFR and heparin, but that in the absence of heparin less stable complexes between FGF and the FGFR can still result in partial receptor activation.

Tissue-specific differences in the structure of heparan may further modify the activity of FGF

Comparison of the amino acid residues in the heparin/HS binding region of FGF show that none are completely conserved throughout the entire family[18], suggesting that different FGFs have different affinities and specificities for unique HS sequences. In support of this, the receptor-specificity of FGF4 for FGFR1 and FGFR2 was found to depend on the concentration of heparin.[48] Studies on the structure of HS isolated from different tissues and cell types have identified variations in the pattern of O-sulfation superimposed on a largely invariant pattern of N-sulfation. These tissue-specific HS fragments of defined sequence have been shown to differentially activate FGFs 1, 2 and 4.[41] Other studies have shown that 2-O-sulfate is required for FGF to bind the FGFR with high affinity but that additional 6-O-sulfation is required for receptor activation (reviewed in Refs. 18, 49). Together these observations suggest a model in which tissue-specific patterns of HS O-sulfation and the local concentration of HS can regulate the activity and specificity of FGFs (Fig. 1C). Regulation of the tissue-specific activity of 2-O- and 6-O-sulfotransferases could therefore be a mechanism to further modulate the activity or change the receptor specificity of FGFs.

In conclusion, multiple layers of regulation control the activity of FGFs. These include tissue-specific expression of ligands and receptors, modulation of binding specificity by alternative splicing of FGFRs and by sequence differences between FGFs and FGFRs, and modulation of binding affinity and specificity by HS molecules with specific patterns of sulfation.

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