Heparan sulfate proteoglycans are essential for FGF receptor signaling during Drosophila embryonic development

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SUMMARY

The Drosophila sugarless and sulfateless genes encode enzymes required for the biosynthesis of heparan sulfate glycosaminoglycans. Biochemical studies have shown that heparan sulfate glycosaminoglycans are involved in signaling by fibroblast growth factor receptors, but evidence for such a requirement in an intact organism has not been available. We now demonstrate that sugarless and sulfateless mutant embryos have phenotypes similar to those lacking the functions of two Drosophila fibroblast growth factor receptors, Heartless and Breathless. Moreover, both Heartless- and Breathless-dependent MAPK activation is significantly reduced in embryos which fail to synthesize heparan sulfate glycosaminoglycans. Consistent with an involvement of Sulfateless and Sugarless in fibroblast growth factor receptor signaling, a constitutively activated form of Heartless partially rescues sugarless and sulfateless mutants, and dosage-sensitive interactions occur between heartless and the heparan sulfate glycosaminoglycan biosynthetic enzyme genes. We also find that overexpression of Branchless, the Breathless ligand, can partially overcome the requirement of Sugarless and Sulfateless for Breathless activity. These results provide the first genetic evidence that heparan sulfate glycosaminoglycans are essential for fibroblast growth factor receptor signaling in a well defined developmental context, and support a model in which heparan sulfate glycosaminoglycans facilitate fibroblast growth factor ligand and/or ligand-receptor oligomerization.

Key words: Drosophila, FGF, Heparan sulfate glycosaminoglycan, Mesoderm, Trachea, Cell migration

INTRODUCTION

The fibroblast growth factors (FGFs) constitute a large family of proteins that act as key intercellular signals in a wide range of developmental and pathological processes (Hanahan and Folkman, 1996; Martin, 1998; Wilkie et al., 1995; Yamaguchi and Rossant, 1995). The biological functions of FGFs include the regulation of cell proliferation, differentiation, survival, motility and tissue patterning. Both biochemical and genetic studies demonstrate that FGFs relay their signals through high affinity transmembrane protein tyrosine kinase receptors (Mason, 1994; Wilkie et al., 1995). By binding to the extracellular domains of these receptors tyrosine kinases (RTKs), FGFs induce the formation of receptor homo- or heterodimers. FGF receptor (FGFR) oligomerization results in RTK transphosphorylation followed by the activation of a Ras-dependent intracellular signal transduction pathway (Fantl et al., 1993).

In addition to its high affinity receptor, biochemical studies indicate that heparin/heparan sulfate proteoglycans (HSPGs) act as low affinity FGF co-receptors that facilitate FGF signal transduction (Mason, 1994; Ornitz et al., 1992; Rapraeger et al., 1991; Schlessinger et al., 1995; Spivak-Kroizman et al., 1994; Yayon et al., 1991). HSPGs are ubiquitous macromolecules that are associated with the cell surface and with the extracellular matrix (Bernfield et al., 1992; David, 1993; Kjellén and Lindahl, 1991; Yanagisawa and Hascall, 1992). HSPGs consist of a protein core to which heparin/heparan sulfate glycosaminoglycan (HS GAG) is attached. The function of HSPGs in FGF signaling is mediated by HS GAG chains which are usually highly sulfated and negatively charged. While the precise mechanism by which HS GAGs participate in FGFR activation remains unclear, a variety of biochemical studies suggest that HS GAGs may stabilize or induce the formation of FGF dimers or a ternary complex composed of ligand plus high and low affinity receptors (DiGabriele et al., 1998; Faham et al., 1996; Herr et al., 1997; Ornitz et al., 1992, 1995; Spivak-Kroizman et al., 1994; Venkataraman et al., 1996; Zhu et al., 1993). Although there is strong in vitro evidence implicating HS GAGs in FGFR signaling, there is as yet no in vivo genetic support for this hypothesis.

Two FGFRs, Heartless (Htl) and Breathless (Btl), have been characterized in Drosophila. Genetic analyses have established...
that each of these receptors has distinct expression patterns and developmental functions during embryogenesis. Htl is expressed uniformly in the early embryonic mesoderm where it is required for the normal dorsolateral migration of mesodermal cells following gastrulation (Beiman et al., 1996; Gisselbrecht et al., 1996; Michelson et al., 1998b; Shishido et al., 1993, 1997). Htl expression is modulated after mesoderm migration is complete, and its continued activity is essential for the specification of particular cardiac and muscle cell fates (Carmena et al., 1998; Michelson et al., 1998b). Btl is expressed in the tracheal system as well as in a subset of cells in the CNS midline (Klämbt et al., 1992; Shishido et al., 1993). Both of these cell types depend on Btl for their specific patterns of migration. For example, in btl mutant embryos, the primary specification of tracheal cells is normal but these cells fail to migrate properly, leading to profound defects in the formation of the tracheal tree (Klämbt et al., 1992). Btl also is required for the determination of specialized cells at the ends of primary tracheal branches that initiate the formation of higher order branching (Lee et al., 1996; Reichman-Fried and Shilo, 1995). While the ligand for Htl has not yet been identified, the Btl ligand is encoded by branchless (bnl; Sutherland et al., 1996).

Mutations in bnl are associated with defects in tracheal morphogenesis that are virtually identical to those seen in btl mutants. Bnl is expressed dynamically in clusters of cells that are positioned so as to guide the outgrowth and subsequent branching of neighboring tracheal cells. Ectopic expression of Bnl can redirect tracheal cell outgrowth and branch formation, substantiating the hypothesis that spatially localized activation of the Btl receptor is necessary for normal tracheal morphogenesis (Lee et al., 1996; Sutherland et al., 1996). The characterization of FGFRs and their ligands by a combination of genetic as well as molecular approaches in Drosophila provides a very useful paradigm for studying the biological functions of FGFs and for identifying other genetic components involved in FGF signaling.

We have recently identified and characterized two Drosophila mutations, sugarless (sgl, also known as kiwi and suppenkasper; Binari et al., 1997; Häcker et al., 1997; Haerry et al., 1997) and sulfatetless (sfl; Lin and Perrimon, 1999) which encode the homologs of UDP-D-glucose dehydrogenase and heparin/heparan sulfate N-deacetylase/N-sulfotransferase, respectively. These enzymes are critical for the biosynthesis and modification of HS GAGs, and the corresponding mutants provide an in vivo model for examining the involvement of these molecules in FGFR signaling. In particular, we have used these mutants to test the hypothesis that HSPGs act as FGF coreceptors by determining whether sfl or sgl null embryos exhibit phenotypes characteristic of the high affinity FGFR mutants, hut and btl, whether sfl and sgl interact genetically with hut, and whether Hut- and Btl-dependent signaling pathways are activated in sfl and sgl null embryos. In this report, we demonstrate that the complete loss of both the maternal and zygotic components of either sfl or sgl expression is associated with profound defects in mesoderm and tracheal cell migration. Moreover, both the Hut- and Btl-dependent activation of MAPK that is seen normally in migrating mesodermal and tracheal cells is undetectable in sfl and sgl mutant embryos. These findings provide the first genetic evidence that HS GAGs are essential for signaling by FGFRs during embryonic development.

MATERIALS AND METHODS

Drosophila strains

The following Drosophila strains were employed: hut^{AB42}, hut^{YY262} (Gisselbrecht et al., 1996; Michelson et al., 1998a), sfl^{303844} (Lin and Perrimon, 1999), sgl^{308310} (Häcker et al., 1997), hut^{LG19} (Klämbt et al., 1992), bnl^{P3} (Sutherland et al., 1996), wg^{CX4} (Bejsovec and Wieschaus, 1993; van den Heuvel et al., 1993), 69B-Gal4(Brand and Perrimon 1993), twi-Gal4 (Greig and Akam, 1993), UAS-Bnl (Sutherland et al., 1996), UAS-Hut^{40} (Michelson et al., 1998a) and trh-lacZ (Wilk et al., 1996). hut^{AB42}, sfl^{303844}, sgl^{308310}, hut^{LG19}, bnl^{P3} and wg^{CX4} are all null alleles by genetic and/or molecular criteria. Oregon R was used as a wild-type reference strain.

Generation of females with sfl and sgl germline clones

Females with germline clones were generated using the autosomal ‘FLP-DFS’ technique (Chou and Perrimon, 1996). Briefly, virgin females of the genotype sfl (or sgl) FRT^{24}TM3, Sb were mated with males of the genotype y w FLP^{23}/+, FRT^{23}P^{ovo D1}/TM3, Sb. The resulting progeny were heat shocked at 37°C for 2 hours during the larval stages of development, and y w FLP^{23}/+; sfl (or sgl) FRT^{23}/FRT^{23} P^{ovo D1/1} females carrying sfl (or sgl) homozgyous germline clones were selected. Such females were mated to sfl (or sgl)TM3-fz;flacZ males, and maternal/zygotic null embryos were identified by the absence of lacZ expression.

Antibody staining

Fixation of embryos, antibody staining and embryo sectioning were performed as described by Gisselbrecht et al. (1996); Michelson et al. (1998a); Patel (1994). Anti-tracheal lumen antibody mAb2A12 was obtained from the Developmental Studies Hybridoma Bank, anti-β-galactosidase antibody from Cappel and Promega, and anti-diphospho-MAPK antibody from Sigma.

Ectopic expression experiments

Targeted ectopic expression was accomplished using the Gal4/UAS system (Brand and Perrimon 1993). Chromosomes bearing Gal4 and UAS insertions were combined with appropriate mutations using standard genetic crosses. Mesodermal and ectodermal expression were achieved with twi-Gal4 and 69B-Gal4 lines, respectively, in both wild-type and mutant genetic backgrounds.

Quantitation of mesodermal phenotypic severity

The hypomorphic hut^{YY262} mutation was recombined with null alleles of sfl and sgl. Embryos from stocks containing these recombinant chromosomes maintained over a nonpole (Lin and Perrimon, 1993) provides an in vivo model for examining the involvement of these molecules in FGFR signaling. In particular, we have used these mutants to test the hypothesis that HSPGs act as FGF coreceptors by determining whether sfl or sgl null embryos exhibit phenotypes characteristic of the high affinity FGFR mutants, hut and btl, whether sfl and sgl interact genetically with hut, and whether Hut- and Btl-dependent signaling pathways are activated in sfl and sgl null embryos. In this report, we demonstrate that the complete loss of both the maternal and zygotic components of either sfl or sgl expression is associated with profound defects in mesoderm and tracheal cell migration. Moreover, both the Hut- and Btl-dependent activation of MAPK that is seen normally in migrating mesodermal and tracheal cells is undetectable in sfl and sgl mutant embryos. These findings provide the first genetic evidence that HS GAGs are essential for signaling by FGFRs during embryonic development.

RESULTS

The genetics of HSPG biosynthesis in Drosophila

In a screen to characterize the maternal effects of zygotic lethal mutations (Perrimon et al., 1996), two mutants, sfl and sgl, were isolated on the basis of their abnormal embryonic segmentation phenotypes. Embryos lacking maternal germline-derived sfl or sgl activity, as well as the paternally...
derived zygotic activity of these genes (referred to hereafter as sfl or sgl null embryos), exhibit a cuticle phenotype similar to that of the wingless (wg) segment polarity mutant (Binari et al., 1997; Hacker et al., 1997; Haerry et al., 1997; Lin and Perrimon, 1999). sgl encodes a homolog of bovine UDP-glucose dehydrogenase (Hempen et al., 1994) which catalyzes the conversion of UDP-D-glucose to UDP-D-glucuronic acid, an essential substrate for GAG biosynthesis. Consistent with a critical role for Sgl in the synthesis of proteoglycans in Drosophila, prior biochemical experiments have established that Syndecan and Dally/Glypican lack GAG chains in sgl null embryos (Haerry et al., 1997) or homozygous sgl zygotic mutant third instar larvae (Tsuda et al., 1999), respectively. Moreover, injection of either UDP-glucuronic acid or heparan sulfate into sgl null embryos rescues the wg-like segment polarity defect, and injection of heparinases I and III (but not chondroitin ABC) into wild-type embryos phenocopies loss of sgl function (Binari et al., 1997). These findings provide strong evidence that Sgl is essential for the biosynthesis of HS GAG chains which, in turn, are required for Wg signaling.

Drosophila Sfl (Lin and Perrimon, 1999) has 51% and 53% amino acid identity to rat and mouse heparin/heparan sulfate N-deacetylase/N-sulfotransferase, respectively (Eriksson et al., 1994; Hashimoto et al., 1992; Orellana et al., 1994). This enzyme catalyzes the N-deacetylation and N-sulfation of polymerized heparan (as opposed to chondroitin or deramatan) GAGs, the key step that initiates further GAG modifications (for review see Kjellén and Lindahl, 1991). Since the sulfation and epimerization of HS GAGs provide structural identity as well as the negative charge that is critical for their interaction with proteins, loss of Sfl activity will result in the generation of unmodified HS GAGs, thereby impairing normal HSPG functions. Indeed, similar to the case in sgl mutants (Tsuda et al., 1999), the HS GAG-modified Dally protein is markedly reduced in sfl zygotic mutant larvae, indicating that, in the absence of Sfl activity, HS GAG biosynthesis is abnormal (Lin and Perrimon, 1999). Together with the strong wg-like segment polarity phenotype associated with loss of sfl function, these results provide compelling evidence that Sfl is essential for the production of proteoglycans involved in Wg signaling (Lin and Perrimon, 1999). Moreover, the specificity of Sfl for heparan-containing GAGs distinguishes the type of GAG chain that participates in this signaling pathway. In summary, phenotypic analyses of sfl and sgl mutants can be used to study the roles of HSPGs in normal developmental processes.

sfl and sgl mutants phenocopy the mesoderm migration defect associated with loss of htl function

After invaginating through the ventral furrow at gastrulation, Twist- (Twi) expressing mesodermal cells migrate along the ectoderm in a dorsolateral direction. By late stage 9, the mesoderm is composed of an inner monolayer of cells that extends from the ventral midline to the dorsal edge of the ectoderm (Bate, 1993; Leptin, 1995; Fig. 1A,E). In htl mutant embryos, gastrulation is normal but mesoderm migration fails to occur properly, resulting in an irregular dorsal margin of Twi-positive cells and a relative accumulation of these cells in ventral and lateral positions (Beiman et al., 1996; Gisselbrecht et al., 1996; Shishido et al., 1997; Michelson et al., 1998b; Fig. 1B,F). A similar phenotype occurs in sfl and sgl null embryos (Fig. 1C,D,G,H). Of note, mesoderm migration is normal in wg mutant embryos (data not shown), suggesting that this effect of sfl and sgl is not due to an influence on Wg signaling, as is the case for the segmentation effects of these genes (Binari et

Fig. 1. sfl and sgl are required for the dorsolateral migration of the embryonic mesoderm, a requirement that is partially bypassed by constitutive Htl signaling. (A-D) Ventral views of late stage 9 embryos of the indicated genotypes immunostained for Twi expression. mat+zyg; an embryo from a germline clone female that has also inherited a mutant paternal chromosome. These embryos therefore lack both the maternal and zygotic functions of sfl or sgl. (E-H) Transverse sections of Twi-stained early stage 10 embryos of the indicated genotypes. Whereas Twi-positive mesodermal cells have reached the dorsal ectoderm in wild type, mesoderm migration fails to occur properly in null htl as well as in maternal/zygotic null sfl and sgl embryos. (I-L) twi-Gal4-mediated ectopic expression of a constitutively activated form of Htl partially rescues the mesoderm migration defects of null htl and maternal/zygotic null sfl and sgl embryos, but has no effect on migration in an otherwise wild-type genetic background (I).
al., 1997; Häcker et al., 1997; Haerry et al., 1997; Lin and Perrimon, 1999).

The finding that mesoderm migration depends on sfl and sgl, as well as on htl, raises the possibility that HS GAG biosynthesis is required for signaling by the Htl FGFR. If, as has been suggested, HS GAGs participate in the activation of FGFRs (Ornitz et al., 1992; Rapraeger et al., 1991; Schlessinger et al., 1995; Yayon et al., 1991), then a constitutively active form of Htl should bypass the requirement of sfl and sgl for mesoderm migration. We tested this hypothesis by targeting the expression of activated Htl to the mesoderm of sfl or sgl null embryos using the Gal4/UAS system (Brand and Perrimon, 1993). We previously reported the construction of such an activated receptor in which the extracellular domain of wild-type Htl is replaced by the dimerization domain of the bacteriophage λ cI repressor (Michelson et al., 1998a). As with other RTKs, this manipulation generates constitutive, ligand-independent receptor activity (Lee et al., 1996; Michelson et al., 1998a; Queenan et al., 1997). In otherwise wild-type embryos, twi-Gal4-mediated ectopic expression of activated Htl has no significant effect on the migration of mesodermal cells (Michelson et al., 1998a; Fig. 1I). However, activated Htl is able to weakly restore mesoderm migration in a null htl mutant (Michelson et al., 1998a; Fig. 1J). Similarly, activated Htl partially rescues the migration defect of sfl and sgl null embryos (Fig. 1K,L). Only partial rescue is seen in all mutant backgrounds due to the relatively weak constitutive activation of Htl that is achieved by spontaneous dimerization (Michelson et al., 1998a). In addition, constitutive Htl does not reproduce the graded activity of this receptor that occurs during normal mesoderm migration (Gabay et al., 1997b; Michelson et al., 1998a). The timing of constitutive Htl expression induced by twi-Gal4 is unlikely to be a contributing factor since the equivalent expression of wild-type Htl completely rescues a null htl allele (Michelson et al., 1998b). In summary, these
genetic epistasis experiments suggest that Htl acts downstream of Sfl and Sgl in migrating mesodermal cells.

If HS GAGs participate in the activation of Htl, then sfl and sgl should exhibit dosage-sensitive genetic interactions with htl. We investigated this possibility using a quantitative assay that is based on the expression of even skipped (eve) in dorsal mesodermal progenitor cells (Buff et al., 1998; Carnena et al., 1998; Frasch et al., 1987; Michelson et al., 1998a,b). The development of these Eve-expressing cardiac and somatic muscle cells depends on Htl for both mesoderm migration and cell fate specification. In wild-type embryos, Eve is found in segmentally repeated groups of cells that are confined to the dorsal mesoderm (Fig. 2A). All such cells are missing in a null htl mutant (Gisselbrecht et al., 1996). However, in a htl hypomorph, mesoderm migration and subsequent cell fate specification are only partially disrupted, permitting some dorsal Eve expression to occur (Fig. 2B; Michelson et al., 1998b). In embryos in which only the zygotic activity of sfl or sgl is absent, Eve expression is entirely normal, reflecting the strong maternal contribution of these genes (Fig. 2C and data not shown). However, when complete zygotic loss of sfl or sgl is combined with homozgyosity for the htl hypomorphic allele, the severity of the Eve phenotype is enhanced (Fig. 2D).

Quantitation of the number of Eve-positive hemisegments in htl, sfl, sgl, htl sfl and htl sgl embryos demonstrated that the interactions between htl and both sfl and sgl are highly significant (Fig. 2E; P<10\(^{-5}\)). These results, combined with the above findings that htl is epistatic to sfl and sgl, are consistent with the hypothesis that HS GAGs are essential for Htl activation during mesoderm migration in the Drosophila embryo.

**sfl and sgl are required for Btl-dependent tracheal cell migration**

Given the genetic evidence that Sfl and Sgl are required for Htl FGFR signaling in the mesoderm, we next determined whether
HS GAG biosynthesis is also involved in signaling by Bnl and Btl during trachea development. Since Wg function is compromised in sgl and sfl null mutant embryos, and wg loss-of-function itself leads to abnormal tracheal morphogenesis secondary to an accompanying segmentation defect (see below), it is difficult to solely correlate the tracheal cell migration phenotype of sgl and sfl null mutants with Bnl/Btl signaling. We therefore analyzed tracheal cell migration in embryos deficient only in the zygotic functions of sfl and sgl. Segmentation and the expression of Engrailed are normal in such embryos, indicating that Wg signaling is unaffected (Perrimon et al., 1996; Häcker et al., 1997; Lin and Perrimon, 1999).

The tracheal system of the Drosophila embryo forms by a sequential series of branching steps that can be visualized by following the expression of an enhancer trap in the trachealess (trh) gene (Isaac and Andrew, 1996; Wilk et al., 1996; Fig. 3A). In stage 13 embryos homozygous for loss of the zygotic functions of either sfl or sgl, the early steps in tracheal branching are significantly perturbed (Fig. 3B,C). By late stage 15, wild-type embryos have developed an extensive tracheal network in which the dorsal and lateral trunk branches have fused and additional primary and secondary branches have formed (Samakovlis et al., 1996; Fig. 3D). In either sfl or sgl zygotic mutants, tracheal branch formation is incomplete, as revealed by the presence of large gaps in the dorsal and lateral trunks, as well as stalled ganglionic branches (Fig. 3E,F). The penetrance of this phenotype is incomplete and the expressivity is variable in both sfl and sgl zygotic mutants; 16% of sfl (n=245) and 7% of sgl (n=198) zygotic mutant embryos exhibit some degree of abnormal tracheal morphogenesis, ranging from one to all segments having breaks in the dorsal trunk. In contrast, in btl and bnl null mutant embryos, tracheal cell migration is more severely affected and virtually no branches form from the initial tracheal invaginations (Klämbt et al., 1992; Sutherland et al., 1996; Fig. 3G). The partial disruption of the tracheal tree seen with zygotic loss of sfl and sgl is reminiscent of the defects observed in hypomorphic mutants of btl, bnl and heartbroken (hbr)/dof/stumps, a gene that encodes a specific effector of FGF receptor signaling (Klämbt et al., 1992; Michelson et al., 1998a; Sutherland et al., 1996; Vincent et al., 1998; Imam et al., 1999). The relatively weak zygotic sfl and sgl tracheal phenotypes are most likely due to partial rescue by the maternal expression of these genes, as previously noted for their mesodermal activities. The more severe tracheal defects that occur in sfl and sgl null embryos are consistent with this suggestion (see below). In summary, the present findings implicate Sfl- and Sgl-dependent HS GAG biosynthesis in signaling by the Btl FGFR.

**Htl- and Btl-dependent MAPK activation depends on sfl and sgl**

Htl and Btl are RTKs that transduce their intracellular signals by the conserved Ras/MAPK cascade (Cobb and Goldsmith, 1995; Seger and Krebs, 1995). As a result, RTK activity can be visualized in developing tissues with an antibody specific for the diphosphorylated, activated form of MAPK (Gabay et al., 1997a,b). Using this approach, diphospho-MAPK is observed at the leading edge of the migrating mesoderm in wild-type embryos (Gabay et al., 1997b; Michelson et al., 1998a; Vincent et al., 1998; Fig. 4A). In either htl or hbr/dof/stumps mutants, this expression of diphospho-MAPK is undetectable (Michelson et al., 1998a; Vincent et al., 1998; Fig. 4B). Similarly, the Htl-dependent mesodermal localization of diphospho-MAPK is below the level of detection in both sfl and sgl null embryos (Fig. 4C,D). In contrast, epidermal growth factor receptor (EGFR)-dependent MAPK activation in the ventral epidermis and amnioserosa does not require either sfl or sgl, consistent with the specific involvement of these genes in FGFR signaling.

During stage 11, MAPK is activated by Btl in the tracheal pits (Gabay et al., 1997b; Fig. 4E). As expected, this expression of diphospho-MAPK is markedly reduced in btl and bnl mutant embryos (Fig. 4F,G). The FGFR-specific signal transducer Hbr/Dof/Stumps also is required for MAPK activation in the tracheal pits (Michelson et al., 1998a; Vincent et al., 1998). In contrast, in wg mutants MAPK activation by Btl is unaffected, although the normal spacing between the tracheal pits is reduced due to the associated segmentation defect (Fig. 4H). As with btl, bnl and hbr/dof/stumps, Btl-dependent MAPK activation is significantly decreased in sfl or sgl null embryos (Fig. 4L,J), whereas the earlier EGFR-dependent expression of...
Overexpression of Bnl partially overcomes the requirement of \( sfl \) and \( sgl \) for tracheal cell migration

It has been suggested that monomeric FGF molecules are capable of self-assembling into dimers and higher order oligomers but, at physiological concentrations, require a HS GAG to stabilize this interaction (Sasisekharan et al., 1997; Venkataraman et al., 1996). If this is the case, then elevated levels of the growth factor may at least partially overcome the need for the HS GAG to generate a biological response. We tested this possibility by assessing the effects on tracheal cell migration of overexpressing Bnl, the Btl ligand, in \( sfl \) and \( sgl \) null embryos.

In an otherwise wild-type genetic background, high level ectopic ectodermal expression of Bnl hyperactivates Btl, leading to an inhibition of primary tracheal branching, as well as the overproduction of secondary and terminal branches (Sutherland et al., 1996; Michelson et al., 1998a; Fig. 5A,B). These effects of ectopic Bnl are weakly suppressed in the absence of zygotic \( sfl \) and \( sgl \) functions (Fig. 5C,D). In embryos lacking both the zygotic and maternal components of \( sfl \) and \( sgl \) expression, virtually no tracheal branches are observed (Fig. 5E,F). This correlates very well with the marked reduction in diphospho-MAPK expression that is seen at earlier stages in \( sfl \) and \( sgl \) null embryos (Fig. 4I,J). Moreover, ectopic Bnl in the absence of \( wg \) function leads to a very marked increase in fine tracheal branching, very similar to the effect of ectopic Bnl in wild-type embryos (compare Fig. 5B and J). Thus, the severe tracheal phenotype associated with complete loss of \( sfl \) or \( sgl \) function primarily is attributable to an involvement of HS GAGs in Btl rather than in \( Wg \) signaling. The ability of Bnl overexpression to partially bypass the requirement for \( Sfl \) and \( Sgl \) is therefore consistent with a role for HS GAGs in stabilizing or facilitating FGF self-association (Zhu et al., 1993; Ornitz et al., 1995; Faham et al., 1996; Venkataraman et al., 1996; Sasisekharan et al., 1997; DiGabriele et al., 1998). Such a HS GAG-FGF dimer complex would, in turn, facilitate the dimerization of high affinity FGFRs, a prerequisite for receptor activation and the transmission of intracellular signals.

DISCUSSION

We have shown that two enzymes involved in the biosynthesis of HS GAGs are essential for signaling by both of the known \emph{Drosophila} FGFRs, \( Htl \) and \( Btl \). Loss of either \( sfl \) or \( sgl \) function leads to defects in the migration of mesodermal and tracheal cells during embryogenesis. In addition, \( Htl \) - and \( Btl \)-dependent activation of MAPK is markedly reduced in \( sfl \) and \( sgl \) null embryos. Taken together, these findings provide the first genetic evidence that HSPGs play a central role in FGF signaling in a well-defined developmental context. A similar genetic approach has been used to establish that HSPGs are critical components of \( Wg \) signaling in \emph{Drosophila} (Binari et al., 1997; Häcker et al., 1997; Lin and Perrimon, 1999; Tsuda et al., 1999).

Several mechanisms have been proposed for how HSPGs participate in FGFR signaling. In one model, the binding of diphospho-MAPK in the tracheal placodes is unaffected in each of these mutants (Michelson et al., 1998a; data not shown). These in situ patterns of MAPK activation provide direct evidence that \( Sfl \) and \( Sgl \) are required for signaling by the two \emph{Drosophila} FGFRs, \( Htl \) and \( Btl \), independent of the requirement for HS GAGs in \( Wg \) function.
FGF to abundant but low affinity HS GAGs on the cell surface limits the free diffusion of the ligand, thereby increasing its local concentration and the probability that it will interact with less abundant, high affinity signaling receptors (Schlessinger et al., 1995). Other studies have identified distinct HS GAG binding sites on both FGF (Blaber et al., 1996; Eriksson et al., 1991; Zhang et al., 1991) and high affinity FGFRs (Kan et al., 1993; Pantoliano et al., 1994), suggesting that the latter two components form a ternary complex with a proteoglycan. Direct interaction between the proteoglycan and FGFR, in addition to growth factor binding, may therefore be required for maximal receptor activation. HS GAGs also may promote the formation of FGF dimers or higher order oligomers, thereby facilitating FGFR dimerization and activation (Zhu et al., 1993; Spivak-Kroizman et al., 1994; Ornitz et al., 1995; Faham et al., 1996; Digabriele et al., 1998).

A variation of the last model proposes that FGF monomers are capable of self-associating, a process that is stabilized by HS GAGs (Venkataraman et al., 1996; Herr et al., 1997; Sasisekharan et al., 1997). This hypothesis predicts that elevated levels of FGF should compensate at least in part for a loss of dimer stabilization mediated by HS GAGs. The ability of Bnl overexpression to induce some tracheal branching in the complete absence of Sfl and Sgl activities is consistent with this last possibility, although it does not rule out an additional involvement of HS GAGs or the core protein of a proteoglycan in some other aspect of Btl activation. In the case of human FGF-2, self-association in the absence of HS GAGs has been observed by mass spectrometry and biochemical assays at physiological concentrations of ligand. In addition, elevated FGF-2 levels exert biological effects on tissue culture cells that fail to synthesize HS GAGs (Davis et al., 1999). Our Bnl overexpression experiments are in agreement with the latter data for FGF-2. Together, these findings support the proposal that HS GAGs function to stabilize the FGF dimers or higher order oligomers that are formed by a self-association mechanism (Venkataraman et al., 1996; Sasisekharan et al., 1997). Interestingly, ectopic expression of ligand also is able to overcome the requirement of HS GAGs in Wg signaling, although in this case the HS GAGs may be more important for increasing the local concentration of growth factor at the cell surface than for facilitating ligand dimerization (Häcker et al., 1997; Lin and Perrimon, 1999).

Under normal conditions, the concentrations of FGFs may be limiting, necessitating the presence of HS GAGs to augment or stabilize ligand dimerization and subsequent FGFR activation. This is of particular significance for Btl signaling since bnl, which encodes its ligand, is known to be haploinsufficient (Sutherland et al., 1996), and overexpression of Bnl partially bypasses the requirement for sfl and sgl in the promotion of tracheal branching. Thus, HS GAGs may ensure that a requisite FGFR activation threshold is surpassed when the amount of available ligand is normally low. Such a mechanism could additionally expand the sensitivity or spectrum of responses that can be achieved by small localized

![Fig. 5. Overexpression of Bnl partially rescues tracheal cell migration in sfl and sgl maternal/zygotic null mutant embryos. Lateral views of late stage 15 embryos immunostained with an antibody against a tracheal lumenal antigen. (A) Wild-type tracheal pattern. In B,C,D,G,H and J Bnl was ectopically expressed throughout the ectoderm of embryos of the indicated genotypes using the 69B-Gal4 driver. (B) Tracheal branching is markedly perturbed by ectopic Bnl in an otherwise wild-type embryo. Primary branching is suppressed and an overabundance of secondary and terminal branches is induced. (C,D) In embryos lacking only the zygotic component of sfl or sgl, the effects of ectopic Bnl are weakly blocked. (E,F) Virtually no tracheal branching occurs in maternal/zygotic null sfl and sgl embryos. (G,H) Some tracheal branching is recovered in maternal/zygotic null sfl and sgl embryos in which Bnl is ectopically expressed at high levels. (I) wg mutants develop an extensive tracheal network which has an abnormal pattern due to the associated segmentation defect. (J) Ectopic Bnl in a wg mutant background leads to an overproduction of fine tracheal branches, much as occurs in wild type (compare with E).]

Role of HSPGs in FGF signaling
differences in growth factor concentrations. This is particularly relevant to Bnl which is expressed in a highly dynamic pattern during normal tracheal morphogenesis, a pattern that, when perturbed, leads to severe defects in tracheal outgrowth (Sutherland et al., 1996). Local differences in Btl activity also dictate the sites at which secondary branches normally form, a process to which HSPG regulation might contribute by generating a zone of cells that are highly responsive to Bnl in the vicinity of the ligand signaling center (Hacohen et al., 1998). Similarly, HSPG-mediated ligand dimerization could play a role in the graded activation of Htl that occurs during embryonic mesoderm migration (Gabay et al., 1997b). Modulation of RTK signaling strength also has been implicated in the generation of mesodermal progenitor identities (Buff et al., 1998).

There are two well characterized HSPGs in Drosophila, Dally, a Glypican-like cell surface molecule that has been implicated in both Decapentaplegic and Wg signaling (Nakato et al., 1995; Lin and Perrimon, 1999; Tsuda et al., 1999), and a transmembrane proteoglycan related to the vertebrate Syndecan family (Spring et al., 1994). It has been suggested that syndecans participate in signaling by vertebrate FGFRs (Bernfield et al., 1992; Dealy et al., 1997; Steinfeld et al., 1996), although other HSPGs may also be involved in this process (Aviezer et al., 1994, 1997; Sherman et al., 1998; Steinfeld et al., 1996). It is also possible that different HSPGs could be specific for particular FGF ligand-receptor combinations in individual tissues or at distinct developmental stages. Genetic analysis in Drosophila should provide a useful approach for addressing these important questions.

The present findings provide new insight into the mechanisms that regulate FGFR signaling in vivo. A more complete understanding of the function of HSPGs in Htl and Btl activation must await definitive identification of the specific proteoglycans involved, as well as structural and biochemical studies of the complexes formed between the high and low affinity receptors together with the corresponding ligands.

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