Role of Heparan Sulfate Proteoglycans in Cell Signaling and Cancer

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I. INTRODUCTION

The correct growth and development of multicellular organisms depends on the reception of numerous extracellular signals that activate various signal transduction cascades within the target cells. The activity of these pathways, such as the receptor tyrosine kinases (RTKs), transforming growth factor-β (TGF-β), Wnts, and Hedgehogs (Hh), are usually regulated by the binding of extracellular ligands to their transducing receptors. Aberrant regulation of these pathways has been linked to many human cancers. Although we have a fair understanding of the structure of these signaling pathways downstream of the receptors, we are only beginning to understand the complexity of the regulatory mechanisms that operate at the extracellular level. Recently, it has become clear that heparan sulfate proteoglycans (HSPGs), a diverse group of cell surface and extracellular matrix proteins, play a key role in modulating a wide range of signaling pathways at this level (Perrimon and Bernfield, 2000).

HSPGs are composed of a protein core modified on specific serine residues by the addition of heparan sulfate (HS) glucosaminoglycans (GAGs) synthesized in the Golgi (Fig. 1; Salmivirta *et al.,* 1996). The HS-GAGs are

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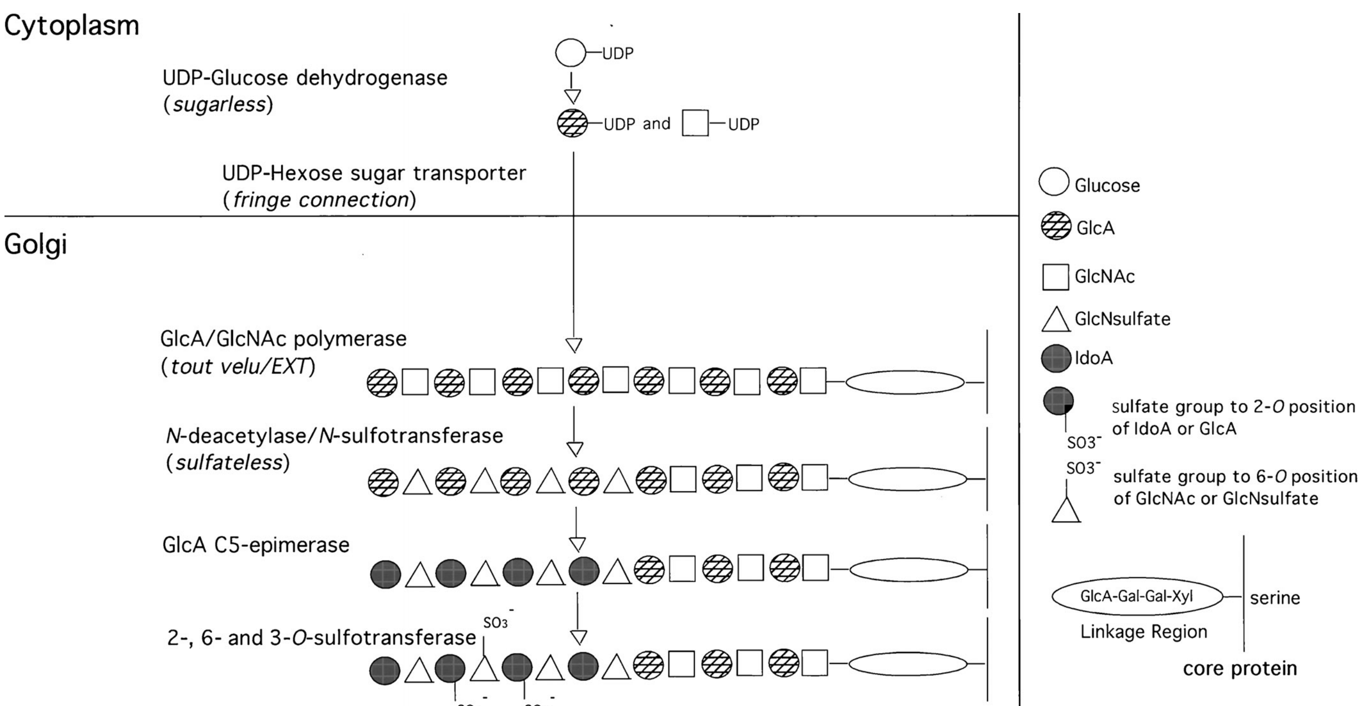

Fig. 1 HSPGs biosynthesis. The substrates for HS biosynthesis, UDP sugars, are synthesized in the cytoplasm and transported into the Golgi by nucleotide sugar transporters. In *Drosophila,* this activity is encoded by *fringe connection* (E. M. Selva, unpublished results). See text for all other details and Salmivirta *et al.* (1996). (Modified with permission from Baeg and Perrimon, 2000.)

defined by10–200 linear repeating disaccharide units of *N*-acetylglucosamine (GlcNAc) and glucuronic acid (GlcA) which are added to the growing HS chain by glycosyltransferases as UDP–sugar substrates. The serine residue of the core protein is attached directly to a xylose–galactose–galactose–GlcA tetrasaccharide linker which serves as the scaffold for the growth of the HS-GAG. Three distinct types of proteins can serve as the HSPG cores: the transmembrane proteins encoded by the *syndecan* genes, the glycosylphosphatidylinositol (GPI) membrane-bound glypicans, and the extracellular matrix secreted perlecan proteins. Extensive postsynthetic modification of the linear HS sugar chains further increases the complexity of HSPGs. Alterations of the linear HS-GAG chains occur in a stepwise manner beginning with N-sulfation of GlcNAc catalyzed by an *N*-deacetylase/ *N*-sulfotransferase, followed by epimerization events and O-sulfation at the C2, C6, and C3 positions of the hexose sugar backbone. Thus, HS sugar chains can be heterogeneously decorated with negatively charged sulfate groups and sugar epimers within any given sugar chain, leading to the potential for a wide range of molecular diversity even among a common protein core. It is thought that the molecular diversity of HSPGs allows for their participation in a wide range of different signaling events and allows them to exert their influence on individual signaling pathways through unique mechanisms.

In the past 3 years, two major advances have been made in understanding the role of HSPGs in development and cancer. First, studies in *Drosophila* have identified many mutations in either the biosynthetic enzymes [e.g., Sugarless (Sgl), Sulfateless (Sfl), and Tout velu (Ttv); Fig. 1] or the protein cores (e.g., syndecans and glypicans). Analysis of the mutant phenotypes has revealed the critical roles of HSPGs in modulating various growth factor signaling pathways. Second, many mutations linked to human cancers have been isolated and shown to correspond to defects in the biosynthesis of HSPGs. Altogether, the studies reviewed here underscore the importance of HSPGs in cell signaling and provide insights into their functions. Possibly, modulating the activity of HSPGs could influence the development of cancers caused by aberrant cell signaling. Thus, HSPGs may constitute important targets for therapeutics to treat some human tumors.

II. HSPGs AND CANCER

The fidelity of HSPG biosynthesis has been shown to be important for the proper activity of many signaling pathways. These include the pathways regulated by the fibroblast growth factor (FGF), TGF-β, Wnt/Wingless (Wg),

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and Hh ligands. In *Drosophila,* as well as in other organisms, these signaling pathways participate in a multitude of proliferative and differentiation events during growth and development. Indeed, aberrant regulation of many of these signaling pathways leads to uncontrolled cell growth associated with the various types of cancers found in vertebrates. For example, ectopic expression of the Wnt-1 oncogene has long been known to lead to mammary tumors in mice (Nusse and Varmus, 1982). Loss-of-function mutations in adenomatous polyposis coli, a downstream effector and negative regulator of the Wnt signaling pathway, are the most common genetic lesions found in colon cancers (Kinzler and Vogelstein, 1996; Polakis, 1997). Mutations in the Hh pathway have also been implicated in human cancers. For example, human carcinomas have been linked to loss-of-function mutations in *patched* (*ptc*). Ptc encodes the Hh receptor and acts as a negative regulator of the pathway such that *ptc* loss-of-function mutations are associated with constitutive signaling (Johnson *et al.,* 1996). Consistent with these findings, Cubitus Interruptus (Ci/Gli), a transcription factor and positive transducer of Hh signaling, is amplified in various types of cancer (Kinzler *et al.,* 1987; Roberts *et al.,* 1989). Finally, TGF-β signaling in mammals acts to both suppress and promote tumorigenesis. Therefore, loss-of-function mutations at any given step in this pathway could result in uncontrolled cell proliferation leading to cancer, whereas aberrant expression of positive regulators of the pathway, such as TGF-β, could result in the same outcome depending on the context. Indeed, loss-of-function mutations in the receptor and downstream effectors of TGF-β signaling have been observed in various cancers, as have high levels of TGF-β expression (Massague *et al.,* 2000).

Mutations in genes involved in the biosynthesis of HSPGs have also been directly implicated in human tumors, consistent with the critical role of HSPGs in regulating the previously mentioned pathways. Two multiple hereditary exostosis (*Ext*) genes have been identified as putative tumor suppressor genes. Loss-of-function mutations in either the *Ext1* or *Ext2* genes are associated with bony outgrowths (exostosis) that can undergo malignant transformation into chondrosarcomas (Hennekam, 1991; Leone *et al.,* 1987) and osteosarcomas (Schmale *et al.,* 1994; Wicklund *et al.,* 1995). *Ext1* and *Ext2* have subsequently been shown to encode a Golgi-localized glycosyltransferase complex required to catalyze the polymerization of UDP–GlcA and UDP–GlcNAc into linear HS chains (McCormick *et al.,* 2000). Furthermore, loss of glypican-3, a GPI-linked HSPG core protein, results in the Simpson–Golabi syndrome, which is characterized by pre- and postnatal overgrowths and a variety of dysmorphisms (Pilia *et al.,* 1996). Finally, a direct potentiating role has been ascribed to HSPGs in Wnt-1-mediated tumorigenesis since mammary hyperplasias were significantly reduced in *wnt-1/syndecan-1* double-knockout mice (Alexander *et al.,* 2000).

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III. FGF SIGNALING AND HSPGs IN D*rosophila*

The FGF signaling cascade is prototypical of the RTK class of signaling pathways. The pathway is initiated by the binding of an extracellular FGF ligand to its cognate tyrosine kinase receptor (FGFR). Ligand binding induces receptor dimerization and subsequent transphosphorylation, which activates a phosphorylation cascade that includes mitogen-activated protein kinase. A large body of work has shown that HS is required for both ligand binding and signal transduction, underscoring the importance of HSPGs in mediating the FGF signal (Ornitz *et al.,* 1992; Rapraeger *et al.,* 1991; Yayon *et al.,* 1991). However, it has only recently been demonstrated through genetic studies in *Drosophila* that HSPGs are required *in vivo* to promote FGF-dependent developmental signaling events (Lin *et al.,* 1999). In the *Drosophila* embryo, homologs of both FGF and FGFR are required for two important developmental events; dorsolateral migration of mesodermal cells (Figs. 2A and 2E) (Beiman *et al.,* 1996; Gisselbrecht *et al.,* 1996; Michelson *et al.,* 1998; Shishido *et al.,* 1993) and tracheal morphogenesis (Lee *et al.,* 1996; Sutherland *et al.,* 1996). Mesodermal migration requires an unknown FGF ligand and the FGFR Heartless (Htl), whereas tracheal migration involves the FGF ligand Branchless (Bnl) and the FGFR Breathless (Btl). In *htl* mutant embryos, mesodermal cells pile up at the ventral midline (Figs. 2B and 2F) (Beiman *et al.,* 1996; Gisselbrecht *et al.,* 1996), a phenotype that can be partially rescued by the expression of an activated form of Htl (Fig. 2J). Embryos that lack both the maternal and zygotic activities of *sgl* or *sfl* exhibit mesodermal defects identical to those observed in *htl* mutant embryos (Figs. 2C, 2D, 2G, 2H, 2K, and 2L). Furthermore, the tracheal defects in *sgl* mutants can be partially rescued by activated Htl, supporting the notion that HSPGs are required upstream of the receptor. The general requirement for HSPG biosynthesis in FGF signaling is also revealed by the observation that zygotic *sgl* and *sfl* mutations yield tracheal branching phenotypes similar to those found in *btl* and *bnl* mutants (Lin *et al.,* 1999). The nature of the protein core that carries the HS-GAG chains is not known, and it will be interesting to determine whether the same protein is involved in both FGF pathways.

The recent crystallographic structure of an FGF–FGFR–HS ternary complex further demonstrates the importance of HSPGs in FGF signaling (Pellegrini *et al.,* 2000; Plotnikov *et al.,* 1999; Schlessinger *et al.,* 2000). Negatively charged heparin (a highly sulfated HS) is bound in a canyon of positive charge (Fig. 3A, shown in blue; see color insert) that is created by the dimerization of FGF receptor–ligand binary complexes. HS forms hydrogen bonds with the FGF–FGFR binary complex and with FGFR from the opposing binary complex [Fig. 3B (see color insert); Plotnikov *et al.,* 1999;

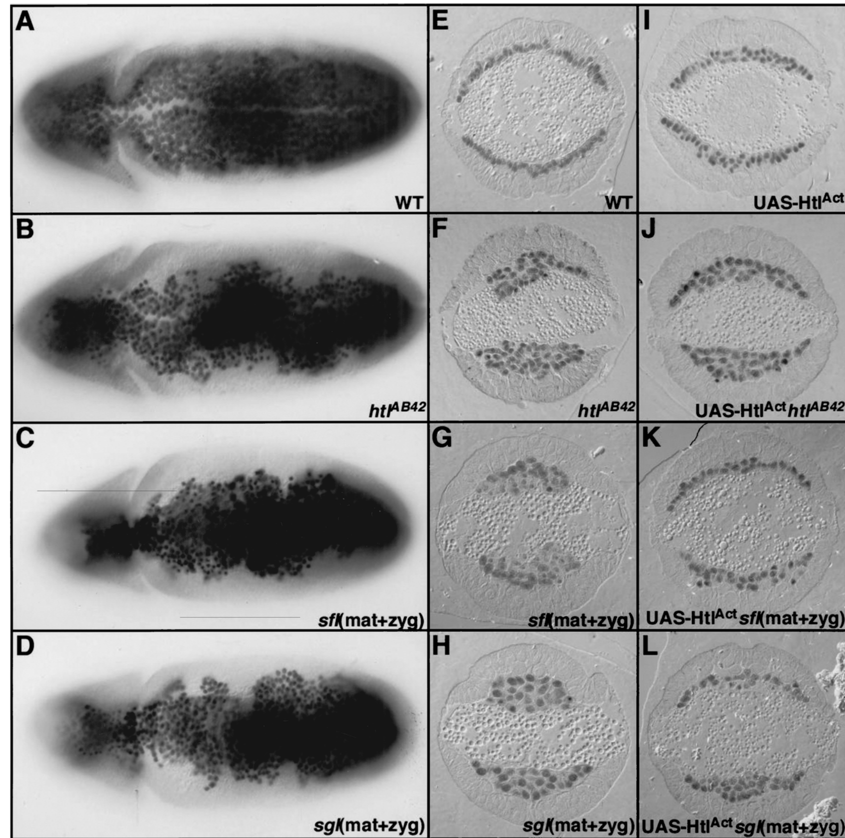

Fig. 2 HSPGs biosynthesis is required for dorsolateral migration of mesodermal cells. (A–D) Ventral view of late stage 9 embryos stained with Twist (Twi), a marker of mesodermal cells. Embryos devoid of both maternal and zygotic expression (germline clone embryos) of *sgl* and *sfl* are identified as mat + zyg. Anterior is to the left. (E–H) Transverse sections of Twi-stained early stage 10 embryos. In wild-type embryos, Twi-positive mesodermal cells have completed their dorsolateral migration, whereas in zygotic null *htl* embryos these cells accumulate at the ventral midline. The same phenotype is also observed for *sgl* and *sfl* germline clone-derived embryos. (I–L) Transverse sections of Twi-stained embryos expressing an activated allele of *htl*. (Reproduced with permission from Lin *et al.,* 1999.)

Schlessinger *et al.,* 2000]. Furthermore, these hydrogen bonds arise primarily from the HS N- and O-sulfate groups (Schlessinger *et al.,* 2000), demonstrating the significance of postsynthetic modification in the specificity of HS molecular interactions. The previous data suggest a model for the role of HSPGs in the activation of FGF signaling *in vivo*. In the signal-receiving cells, HSPGs are likely to both stabilize the FGF–FGFR binary complexes and promote dimerization to yield the active ternary complex. Thus, in the

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context of FGF signaling, the HSPG acts as a coreceptor to facilitate the interaction between the FGF ligands and the FGFR transducing receptors.

IV. Dpp SIGNALING AND HSPGs IN D*rosophila*

In *Drosophila, decapentaplegic* (*dpp*) encodes a member of the TGF-β/ bone morphogenetic protein (BMP) family, and numerous studies have established that the signaling pathway activated by Dpp is evolutionary conserved. The extracellular ligand, Dpp, binds to its heterodimeric types I and II serine/threonine kinase receptor to initiate signaling (Brummel *et al.,* 1994; Penton *et al.,* 1994; Ruberte *et al.,* 1995). The glypican protein, called Dally, has been shown to play a role in Dpp-dependent imaginal disc patterning (Jackson *et al.,* 1997). Through genetic interaction studies, Dally was found to be required downstream of Dpp for signal transduction; when overexpressed, Dally was able to amplify the outcome of Dpp signaling. These observations suggest that Dally can potentiate the activity of Dpp; however, the precise mechanism of HSPG action in Dpp signaling is not understood. Interestingly, in vertebrate cells, cross-linking studies have shown that the HSPG betaglycan interacts with TGF-β and promotes binding to the signaling receptor (Lopez-Casillas *et al.,* 1993). Based on these results, it will be of interest to determine whether vertebrate glypicans can regulate some aspects of TGF-β/BMP signaling.

V. Wg AND Hh SIGNALING AND HSPGs IN D*rosophila*

A member of the Wnt family of secreted glycoproteins has been implicated in many events during *Drosophila* embryogenesis, including segmentation of the epidermis, segmental patterning of the midgut epithelium, formation of the stomatogastric nervous system, neuroblast determination and differentiation, the control of cellular proliferation during Malpighian tubule formation, and generation of epithelial cell type diversity. One function of Wg during embryonic segmentation is to stabilize the expression of both the homeobox gene *engrailed* (*en*) (Ingham and Martinez Arias, 1992; Perrimon, 1994) and the signaling molecule Hh. In the early embryo, *wg* is expressed in stripes of epidermal cells that are immediately adjacent and anterior to cells expressing both *en* and *hh*. The juxtaposition of *en*/*hh* and *wg*-expressing cells is crucial for the formation of alternating bands of naked cuticle and denticles within each segmental unit (Fig. 4; for Figure 4A, see color insert). Expression of both *en*/*hh* and *wg* is first initiated as the result of complex

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B

	wg⁻	hh⁻	sgl⁻/sfl⁻	dlp RNAi
phase 1	+	+	+	+
phase 2: wg	-	-	-	+
phase 2: en/hh	-	-	+	+
phase 3	-	-	-	-
AP reduced denticle lawn	-	-	-	+
denticle lawn	-	-	-	-

Fig. 4 Model for the role of Wg and En/Hh signaling during embryonic segmentation and the concurrent role of HSPGs. (A) See color insert. (B) Table summarizing the effect of mutations that disrupt the Wg or En/Hh signaling cascade. A "+" indicates that a given phase can occur in the absence of the indicated genes, whereas a "−" indicates a block.

regulatory interactions between the pair-rule genes (Fig. 4A, phase 1). Subsequently, the maintenance of both *en/hh* and *wg* expression becomes mutually dependent at stage 8 of embryonic development until early stage 11 (Fig. 4A, phase 2). In *wg* mutant embryos, *en/hh* expression fades from the epidermis, and in *en* and *hh* mutant embryos epidermal *wg* expression disappears due to the absence of Hh signaling (Fig. 4B; Bejsovec and Martinez Arias, 1991; DiNardo *et al.,* 1988; Heemskerk *et al.,* 1991; Martinez-Arias *et al.,* 1988). At a later stage of embryonic development, Wg also signals anteriorly to promote the differentiation of epithelial cells to secrete cuticle that lacks denticle bands (Fig. 4A, phase 3; Sanson *et al.,* 1999). Thus, the absence of either *wg* or *en/hh* function at early stages of embryonic development disrupts overall patterning, generating embryos with a lawn of denticles and reduced size along their anterior–posterior (AP) axis. Loss of Wg activity at later stages of embryogenesis affects long-range patterning and prevents the deposition of naked cuticle to yield a lawn of denticles (Fig. 4B).

Mutations in *sgl, sfl,* and *ttv* were originally identified based on their phenotypic similarities to the *wg/hh* loss-of-function mutations (Bellaiche *et al.,* 1998; Binari and Perrimon, 1994; Häcker *et al.,* 1997; Haerry *et al.,* 1997; Lin *et al.,* 1999; Perrimon *et al.,* 1996). Mutations in these genes that remove both maternal and zygotic activities are associated with segmentation phenotypes that resemble the loss of either Wg or Hh signaling. However, because of the interdependence of the Hh and Wg signaling pathways, it was necessary to analyze the role of these genes in tissues in which they do not regulate each other in order to determine which pathway(s) requires HSPGs for signaling. Such a situation is found in the imaginal disc in which these genes

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control different processes. In the wing disc, Wg organizes patterning along the dorsoventral (DV) boundary and Hh controls AP axis formation (Fig. 5; see color insert).

VI. THE ROLE OF GLYPICANS IN Wg SIGNALING

In the wing imaginal disc, *wg* is expressed at the DV boundary, where it acts as an organizer to control the growth of the wing blade and differentiation of the wing margin. When groups of homozygous *wg* mutant cells are generated in the wing disc (also known as clones), wings develop nicks that overlap the wing margin. A similar phenotype is generated when clones of *sfl* mutant cells are induced (Baeg *et al.,* 2001). However, this is not the case when clones of *sgl* mutant cells are generated, presumably because GlcA, the product of Sgl activity, is able to freely diffuse between cells. The phenotype associated with *sfl* mutant clones, together with its maternal effect phenotype, suggests that HSPGs are required for Wg signaling. Further support for this model derives from the analysis of both the loss- and gain-of-function phenotypes associated with the glypican molecules Dally and Dally-like (Dly) (Baeg *et al.,* 2001; Lin and Perrimon, 1999; Tsuda *et al.,* 1999). For example, *dally* homozygous mutants show a low penetrance of wing nicks at the wing margin. Furthermore, this phenotype can be enhanced by reducing the amount of extracellular Wg, and it can be suppressed by introducing an activated downstream Wg signaling component (Lin and Perrimon, 1999).

How do the HSPGs work in the context of Wg signaling? Interestingly, homozygous *sfl* mutant clones that span the DV boundary of the wing disc did not disrupt the expression (and presumably secretion) of Wg. The distribution of Wg in *sfl* mutant clones is indistinguishable from surrounding wild-type tissue. However, a highly sensitive staining method that detects extracellular Wg showed that Wg was not present at the surface within clones (Baeg *et al.,* 2001). This suggests that HSPGs are required to restrict Wg diffusion and thus may serve to trap extracellular Wg (Fig. 6). In support of this model, *sfl* mutant cells located near wild-type Wg-secreting cells display Wg staining to some extent and thereby show local nonautonomy in mosaic analyses. Another possibility is that HSPGs may increase the local concentration of Wg by preventing its degradation by extracellular proteases. Furthermore, the local nonautonomy of *sfl* mutant clones suggests that the HSPGs are not absolutely required for Wg association with Frizzled, its transducing receptor (Bhanot *et al.,* 1996; Chen and Struhl, 1999). Finally, consistent with this model, overexpression of Dly along the DV boundary yields *wg* loss-of-function phenotype, presumably because Wg is not free to diffuse to its site of action but rather becomes sequestered by the high local concentration of Dly

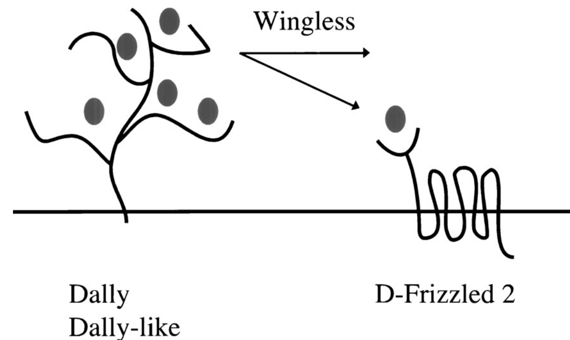

Fig. 6 The influence of HSPGs on Wg signaling in imaginal discs. The binding of extracellular Wg to HSPGs appears to serve two functions. HSPGs facilitate the organization of the extracellular Wg gradient in the wing pouch and promote the interaction of Wg with its signaling receptor, Frizzled (Dfz2).

(Baeg *et al.,* 2001). The proposed role of HSPGs in the context of Wg signaling is distinct from the role of HSPGs in FGF signaling because it does not require an association between the HS-GAG and the ligand/receptor complex.

In the embryo, the segmentation phenotype associated with loss of maternal and zygotic *sgl* and *sfl* suggests that a core HSPG protein must also be important for phase 2 *wg* maintenance and phase 3 Wg-dependent secretion of naked cuticle. The glypican Dally has been proposed to correspond to this protein because weak *dally* alleles and *dally* RNA interference (RNAi) experiments generate embryos reminiscent of partial loss of *wg* function (Baeg *et al.,* 2001; Lin and Perrimon, 1999; Tsuda *et al.,* 1999). Furthermore, *dally* misexpression results in an expansion of the *en/hh* domain (Tsuda *et al.,* 1999). These observations suggest that Dally may be involved in posterior Wg signaling to maintain *en/hh* expression as well as to promote Wg signaling anteriorly. Recently, RNAi experiments with *dly* implicated HSPG in long-range Wg patterning but not during the initiation or maintenance phase (Baeg *et al.,* 2001). Interestingly, both *dally* and *dly* are expressed at high levels anterior to the *wg*-expressing cells (Khare and Baumgartner, 2000; Lin and Perrimon, 1999), which is consistent with these genes playing a major role in the organization of the anterior Wg activity. Together, these data suggest a model in which *wg* and *en/hh* expression is initiated by pair-rule gene expression (Fig. 7; see color insert). Subsequently, maintenance of *wg* and *en/hh* may require the HSPG Dally. Finally, anterior long-range Wg signaling to pattern the naked cuticle may require the action of Dly and, to a lesser extent, Dally. It should be noted that some of these conclusions are not as definitive as one would like since they were based on partial loss-of-function alleles or overexpression experiments. Thus, it will be critical to reexamine these issues once null alleles for either *dally* or *dly* become available.

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VII. HSPGs ARE INVOLVED IN Hh MOVEMENT

Much of what we know about the role of HSPGs in Hh signaling comes from the analysis of the *ttv* mutant phenotype in wing imaginal discs. Ttv/Ext has been shown to play an important role in the movement of Hh from its site of synthesis in the posterior compartment of the wing disc to its site of action in the anterior compartment (Bellaiche *et al.,* 1998). Homozygous mutant clones of *ttv* along the AP boundary show reduced Ptc expression and Ci stabilization, two targets of Hh signaling. Hh is a very unusual protein because it undergoes an autoprocessing event whereby a cholesterol moiety is attached to its N terminus to produce the active ligand (Lee *et al.,* 1994; Porter *et al.,* 1996). The linkage of cholesterol to Hh decreases its solubility and tethers the molecule to the membrane, presumably limiting its long-range diffusion. Recently, *dispatched* (*disp*), which encodes an extracellular membrane protein with a sterol-sensing domain, was found to be required for the release of Hh from sending cells since Hh was retained in clones of *disp* mutant cells (Burke *et al.,* 1999). The current model suggests that Disp is required in the posterior cells to transfer cholesterol-bound Hh to an unidentified anterior compartment HSPG which requires Ttv for its appropriate biosynthesis (Fig. 8). In turn, this complex directly or indirectly transfers Hh to its receptor Ptc to transduce the Hh signal. Whether the *ttv*-dependent HSPG is sufficient for Hh movement within the anterior compartment awaits further investigation. Finally, it is possible that HSPGs also play a more direct role in Hh signaling. In *ttv* embryos that are devoid of both maternal and zygotic gene activity, *wg* expression decays because of defective Hh signaling. Thus, even when Hh signals to immediate neighboring cells, HSPGs may be required for regulation of Ptc by Hh.

Furthermore, Ttv, which encodes a glycosyltransferase by analogy to mammalian Exts, is surprisingly specific to Hh signaling (The *et al.,* 1999). Careful characterization of the *ttv* mutant phenotype failed to reveal a function for Ttv in either the Wg or FGF signaling pathways. This is unexpected because Ttv encodes a polymerase involved in HS-GAG chain biosynthesis; therefore, it should have a phenotype similar to those of either *sgl* or *sfl* mutants (see Fig. 1). The reason why Ttv is specific to Hh signaling is not resolved, and many possibilities that include either qualitative or quantitative models can be considered. For example, one quantitative model is that in the absence of Ttv activity, a reduced amount of HSPGs are synthesized by other Ext enzymes and Hh signaling is much more sensitive to this reduction than either the FGF or Wg pathways. Alternatively, a qualitative model suggests that specific Exts may only modify a subset of protein cores such that in *ttv* mutants the HSPGs involved in Wg and FGF signaling pathways are modified properly, but the HSPGs implicated in Hh signaling are not.

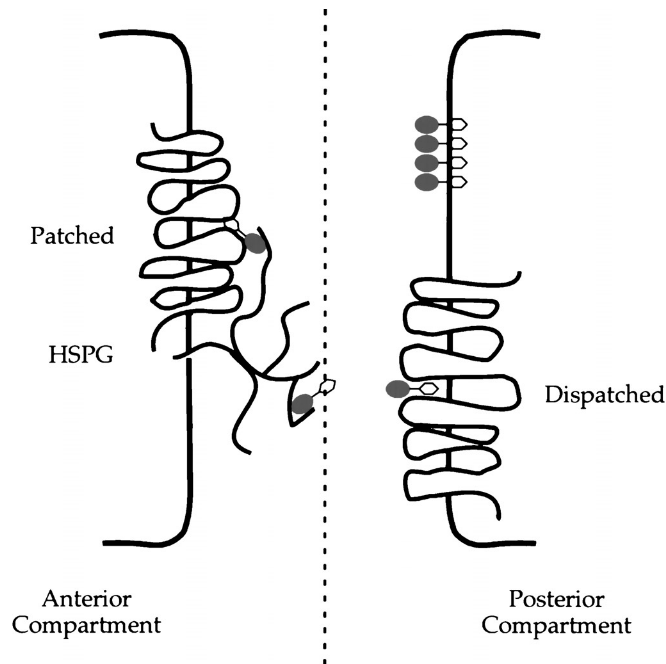

Fig. 8 The role of HSPGs in Hh signaling in imaginal discs. The biologically active Hh ligand is synthesized in the posterior compartment of the wing imaginal disc. The N-terminal region of Hh is linked to a cholesterol moiety which may be localized to microdomains (rafts) within the membrane (Rietveld *et al.,* 1999). Appropriate movement of Hh from the posterior compartment to the anterior compartment requires the activity of Disp with its sterol-sensing domain. Subsequently, Hh requires the glycosyltransferase activity of Ttv for movement within the anterior compartment, presumably by adding HS to an unknown core protein. The movement of Hh from the posterior to the anterior compartment might require a direct transfer between Disp and the HSPG. Once in the anterior compartment, Hh must interact with its receptor Ptc to initiate the downstream signaling cascade; this interaction may also involve a Ttv-dependent HSPG. (Adapted from Ingham, 2000.)

VIII. CONCLUSION

Recent studies of HSPGs have implicated these molecules as key players in regulation of cell–cell communication events. Interestingly, in all the pathways examined to date they appear to positively regulate signaling events. Although the requirement for HSPGs is well documented, the precise mechanisms by which they act remains obscure. An understanding of the molecular interactions between HS-GAGs and growth factors could potentially provide an excellent means to interfere with specific pathway activities and to develop therapeutics that act extracellularly.

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