Glycoconjugate Journal 19, 363–368, 2003

Developmental roles of heparan sulfate proteoglycans in *Drosophila**

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The formation of complex patterns in multi-cellular organisms is regulated by a number of signaling pathways. In particular, the Wnt and Hedgehog (Hh) pathways have been identified as critical organizers of pattern in many tissues. Although extensive biochemical and genetic studies have elucidated the central components of the signal transduction pathways regulated by these secreted molecules, we still do not understand fully how they organize gradients of gene activities through field of cells. Studies in *Drosophila* have implicated a role for heparan sulfate proteoglycans (HSPGs) in regulating the signaling activities and distribution of both Wnt and Hh. Here we review these findings and discuss various models by which HSPGs regulate the distributions of Wnt and Hh morphogens.
Published in 2003.

Keywords: **heparan sulfate proteoglycan, Wnt, Wingless, Hedgehog, *Drosophila***

1. Introduction

During development of multi-cellular organisms, cell fate determination is controlled by the integration of several signaling systems that are initiated by several evolutionarily conserved extracellular molecules. These include members of Wnt, Hedgehog (Hh), transforming growth factor-β (TGF-β) and fibroblast growth factor (FGF) families. Among them, members of the Wnt, Hh and TGF-β families have been shown to function as morphogens; i.e., they are expressed in restricted regions of tissues and can form long-range concentration gradients to specify different cell fates [1,2]. During the past several decades, both biochemical and genetic studies have elucidated the central components of the signal transduction pathways regulated by these molecules. However, less is known about their distribution through tissues and how they act long range to organize pattern.

In the last few years, our laboratory as well as others have conducted *in vivo* functional studies of HSPGs in *Drosophila*. These studies have demonstrated that they play critical roles in regulating the signaling activities of Wnt, Hh, TGF-β and FGF molecules. Further, it has become clear that the distribution of Wnt and Hh morphogens is regulated by HSPGs. This review will focus on the molecular mechanism(s) by which HSPGs regulate the signaling activity and the extracellular distribution of Wnt and Hh molecules. For other aspects of the functions of HSPGs in development, see previous reviews [3–8].

2. HSPGs: A family of evolutionarily conserved molecules

HSPGs are extracellular matrix and cell surface macromolecules that consist of a protein core to which HS glycosaminoglycan (GAG) chains are attached (see Introduction by Silbert and Sugumaran in this issue). These molecules can be classified into several families based on the structure of the protein cores. Glypicans and syndecans are two major cell surface HSPGs, both of which are integral membrane proteins. Glypicans are disulfide-stabilized globular proteins linked to the plasma membrane by a glycosylphosphatidylinositol linkage. Syndecans are transmembrane proteins that contain a highly conserved short [34–38 residue] carboxy-terminal cytoplasmic domain. While glypicans bear exclusively HS chains, syndecans may be decorated with both HS and chondroitin sulfate (CS). Besides syndecan and glypicans, perlecan is a secreted HSPG that is mainly distributed in the extracellular matrix, and has exclusively HS chains [6].

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*This review is dedicated to the memory of Merton Bernfield, our colleague and friend who taught us so much about proteoglycans.

Biosynthesis of HS is initiated in the Golgi at its attachment site to a core protein, which contains between 2 and 4 Ser-Gly sequences. An oligosaccharide primer is first attached to specific serine residues on a proteoglycan core protein. This primer serves as a substrate for the co-polymerases that add alternating linked D-glucuronic acid (GlcA) and N-acetyl-D-glucosamine (GlcNAc) residues, to form chains of about 100 or more sugar units in length. This polymer is subsequently modified through a series of reactions, requiring both N-deacetylation and N-sulfation of GlcNAc units, epimerization of GlcA to L-iduronic acid (IdoA) residues, and O-sulfation at various positions. From these modifications, enormous structural heterogeneity in HS structure can be produced (see introduction by Silbert and Sugumaran in this issue).

Both core proteins and enzymes involved in the biosynthesis of HS are conserved in vertebrates and invertebrates [6,8]. In *Drosophila*, a single *syndecan* gene, two *glypican* genes, and a *perlecan* homolog have been identified [6,8]. So far, functional studies are only available for the two glypican family members, division abnormally delayed (dally) [9], and dally-like (dly) [10,11] (see below). *Drosophila* homologs of most of the vertebrate enzymes involved in HS biosynthesis are also present in the fly genome. These include specific sugar transporters, enzymes involved in the initiation of oligosaccharide primer and specific modifications [3,6]. In the past few years, some of the enzymes that participate in HS biosynthesis have been identified and genetically characterized in *Drosophila* [4,6] see below). Others such as xylosyltransferase [12] and a EXT 3-like glycosyltransferase [13], have been biochemically characterized recently.

3. Identification and isolation of mutations in HS enzymes and core proteins

Mutations in the genes encoding some of the enzymes involved in the biosynthesis of HS were initially identified from our genetic screen designed to characterize the maternal effects of zygotic lethal mutations [14,15]. From this genetic screen, we have isolated several new segment polarity genes that exhibit mutant phenotypes that resemble those associated with loss of either *wingless* (*wg*) or *hh* activity. These genes are *sugarless* (*sgl*) [4,16], *sulfateless* (*sfl*) [4,17,18] and *tout velu* (*ttv*) [19,20], which encode *Drosophila* homologs of UDP-D-glucose dehydrogenase, HS N-deacetylase/N-sulfotransferase and HS polymerase EXT-1, respectively. *dally* was isolated from a different genetic screen targeted to obtain mutants affecting cell division patterning in the developing central nervous system of *Drosophila* [9,21]. The identification of mutations in these genes has provided the starting materials for analyzing the function of these HSPGs. Below, we review our analysis of HSPGs in the context of Wnt and Hh signaling. Further, we discuss the various mechanisms by which HSPGs may regulate the signaling activities and protein distributions of the Wg and Hh morphogens.

4. Role of HSPGs in Wg signaling and morphogen gradient formation

4.1. Wg: A morphogen critical in development

Wnt proteins are members of a large family of secreted, cysteine-rich glycoproteins that act as ligands, and stimulate receptor-mediated signal transduction pathways in both vertebrates and invertebrates [22]. All Wnts contain a signal sequence, 22 conserved cysteine residues, and multiple potential N-linked glycosylation sites. Wnt proteins are poorly secreted into the culture medium and are tightly associated with the cell surface and the extracellular matrix. Thus, how Wnt proteins travel from cell to cell to initiate intercellular signaling is of particular interest. The homolog of vertebrate Wnt-1 is encoded by *Drosophila Wg*, which has been implicated as a mediator of positional information in a wide variety of developmental contexts. These include segmentation of the epidermis, patterning of the midgut epithelium, formation of the stomatogastric nervous system, neuroblast determination and differentiation and patterning of imaginal discs [22,23]. Wg can exert both short- and long-range effects during the development of the embryo and imaginal discs. The function of Wg protein has been particularly well explored in embryonic patterning of epidermis and wing patterning during larval development.

Wg acts both as a short-range inducer and a long-range morphogen during differentiation of the embryonic epidermis (Figure 1). Early in embryogenesis at stage 9–10, *wg* is

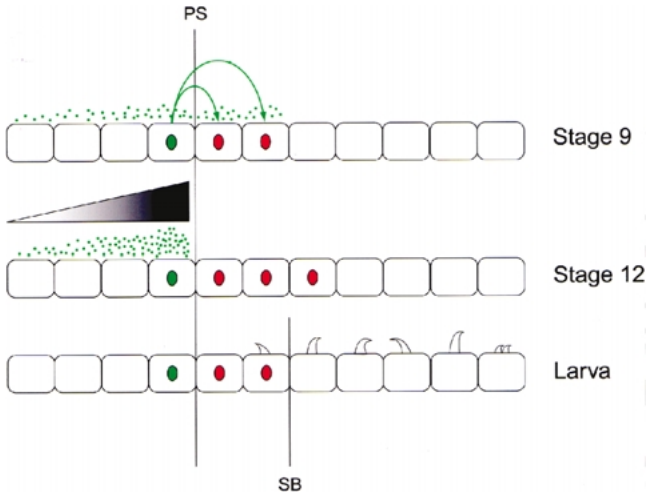

Figure 1. *Roles of Wg signaling during embryonic segmentation in the ventral epidermis.* During embryogenesis, *wg* and *en* are expressed in stripes within each segment. The single row of *wg*-expressing cells is shown in green and *en*-expressing cells are in red. *en* is expressed in cells posterior to the *Wg* stripe. At stage 9, Wg protein is distributed symmetrically and acts short-range posteriorly to maintain *en* expression. At stage 12, Wg protein is mainly distributed anteriorly and forms a morphogen gradient that is required for producing the naked cuticle. The position of the segmental border (SB) and the parasegmental boundary (PS) are indicated.

expressed in stripes of epidermal cells that are immediately adjacent, and anterior, to cells expressing the homeobox gene *engrailed* (*en*). Wg functions at short-range to maintain the expression of *en.* Subsequently, En, through a signaling pathway mediated by Hh is required for the maintenance of *wg* transcription [24]. 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 (Figure 1). At a later stage (stage 12), Wg distributes anteriorly to its expressing cells and forms a concentration gradient required for producing the "naked cuticle" that lacks denticle bands [24,25]. Thus, Wg functions as a long-range morphogen that gives positional information to the anterior cells in front of the source.

The function of Wg as a morphogen is best studied in the wing imaginal disc where it is expressed in a narrow strip of cells at the dorso-ventral compartment border (Figure 2). Wg acts as short-range inducer to specify cell fates at the dorso-ventral boundary. Wg also acts up to 20–30 cell diameters away from its site of synthesis to activate the transcription of downstream target genes in a dose-dependent manner, which is a characteristic of morphogen molecules. The extracellular Wg protein gradient can be visualized on the basolateral surface of the wing imaginal disc epithelium [26]. Wg protein moves rapidly to form a long-range extracellular gradient. It has been proposed that the Wg protein gradient is established by rapid diffusion through the extracellular space, depending on continuous secretion and rapid turnover [1,26].

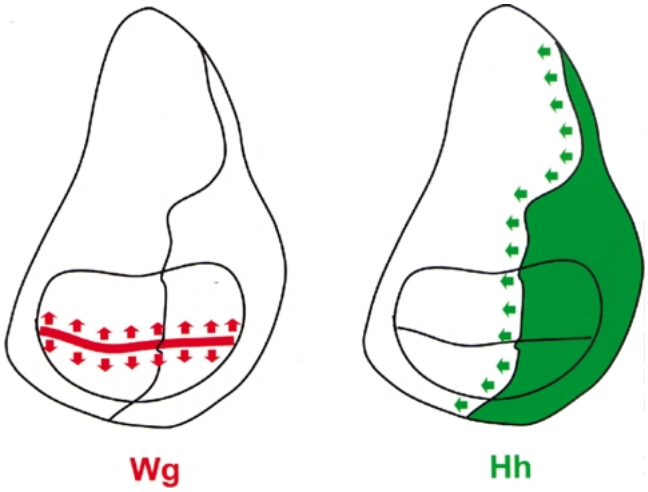

Figure 2. *Roles of Wg and Hh in patterning of the wing imaginal disc.* In a third instar larvae wing imaginal disc, Wg (red) is expressed at the dorso-ventral border of the wing disc (A) and signals dorsally (arrows up) and ventrally (arrows down) to organize patterning. Hh (green) is expressed in the posterior compartment and moves in the anterior compartment of the disc to control anteroposterior axis formation.

4.2. HS is required for Wg signaling and its extracellular distribution

Our genetic analyses have provided evidence for an *in vivo* function of HS in Wg signaling and distribution. Both *sgl* and *sfl* were initially identified and isolated as genes involved in Wg signaling since their mutants exhibit defects of cuticle patterning similar to that of *wg* mutant [16,17]. In *sgl* and *sfl* mutant embryos, we also observed other defects associated with Wg-dependent developmental processes including the development of the somatogastric nervous system and midgut constriction [16,17]. Further, we have obtained genetic evidence for the requirement of HS in both Wg short- and long-range signaling in wing imaginal disc [17]. In the wing discs homozygous for *sfl* mutant, the expression of the short-range target-gene *neuralized* is abolished, and the expression of the long-range target-gene *distaless* (*dll*) is markedly reduced. Furthermore, analysis of *sfl* provided direct evidence for a role of HS in the distribution of extracellular Wg [11] since in *sfl* mutant clones, the distribution of extracellular Wg is dramatically reduced. Altogether, these results point to a role for HS in most, if not all, of the Wg-dependent developmental processes. Mechanistically, at least one of the mechanisms of the HS action in Wg signaling is to regulate the extracellular distribution of Wg [11].

4.3. *Drosophila* glypicans, Dally and Dly, are involved in Wg signaling

We have demonstrated that the two *Drosophila* glypicans, dally and dly, are involved in Wg signaling. First, hypomorphic dally alleles exhibit some wing margin defects [9], a phenotype reminiscent of partial loss of *wg* activity. The wing defects associated with *dally* mutants can be enhanced by a reduction in Wg protein or a reduction in Wg signaling activity, but can be suppressed by introducing an activated downstream Wg signaling component [17]. Second, we also observed that *dally* mutants can suppress gain-of-function phenotype induced by ectopic expression of the Wg receptor *Drosophila* frizzled 2 (Dfz2). These results suggest that Dally is a major HSPG involved in Wg signaling in wing imaginal disc.

We have also demonstrated that Dally and Dly are required for cuticle patterning in embryogenesis. Disruptions of either *dally* or *dly* by RNA interference (RNAi) in embryos, produced patterning defects in the cuticle that are similar to the defects associated with weak *wg* mutants. Interestingly, more severe cuticle patterning defects were observed in embryos injected with *dly* dsRNA than those injected with *dally* RNAi, suggesting that Dly may play a more important role than Dally in Wg signaling—at least in the ventral epidermis. Furthermore, we also observed that many embryos injected with *dly* dsRNA develop cuticles that lack naked cuticle but which are similar in size to wild-type embryos [11]. Since Wg signaling during late embryogenesis is required for producing naked cuticle (Figure 1), these results suggest that Dly is likely to be involved in Wg signaling at later developmental stages. Consistent with

this, we have observed that *dly* transcripts are enriched in cells anterior to the *wg*-expressing cells [11].

Consistent with our results on the role of Dally and Dly in Wg signaling, recently, in both S. Cohen and K. Basler labs, they identified a gene called *notum* or *wingful*, which encodes a protein to function as a repressor for Wg signaling [27]. It encodes a member of the alpha/beta-hydrolase superfamily, with similarity to pectin acetylesterases, and influences Wg protein distribution by modifying the HSPGs Dally-like and Dally [27], further suggesting important roles of Dally and Dly in Wg signaling.

4.4. Molecular mechanism(s) by which HSPGs may regulate Wg signaling and its distribution

Our genetic analyses in both HS enzymes (*sgl* and *sfl*) and glypicans (*dally* and *dly*) have provided strong evidence for a role of HSPG in Wg signaling. Our results also suggest that *dally* and *dly* are the major HSPGs involved in Wg signaling. In the following section, we propose several mechanisms by which HSPGs may regulate Wg signaling and its distribution.

Concentration model

In this model, the function of HSPG is to increase the local concentration of Wg ligand for its receptor. HSPGs bind to Wg and reduce the dimensionality of Wg ligand diffusion from three to two dimensions. In the absence of HSPGs, the concentration of Wg protein may be lower than its threshold concentration and the efficiency of Wg signaling will be reduced. Several genetic experiments support this model. First, we have observed that Wg signaling is not completely abolished in the formation of somatogastric nervous system and Malpighian tubules [16]. Perhaps, the threshold concentration of Wg signaling required for these tissues is relatively low. Second, in the ventral epidermis, over-expression of ectopic Wg protein can compensate for the loss of Wg signaling to partially rescue the segmentation defects associated with *sfl* and *sgl* [16].

Trapping/retention model

HSPGs are required for trapping/retention of Wg protein in receiving cells. This model is based on the following observations. (1) HSPGs are required for the distribution of extracellular Wg protein in imaginal disc [11]. (2) Ectopic expression of Dly in wing imaginal disc leads to an accumulation of Wg protein [11]. (3) Embryos devoid of Dly by RNAi develop segmentation defects that lack naked cuticle, but with relatively normal sized embryos, suggesting that Dly is involved in long-range Wg patterning required for generating naked cuticle [11]. According to this model, Wg moves through rapid diffusion to receiving cells where Wg is trapped by the abundant HSPGs. HSPGs stabilize Wg by either limiting its diffusion or by preventing it from being degraded by extracellular proteases. We propose that both Dally and Dly are involved in Wg morphogen gradient formation in wing disc since both are expressed in this tissue. It is also worth noting that both *Drosophila* syndecan and perlecan are expressed in wing disc (Lin et al. unpublished result) raising the possibility that these molecules contribute to formation of the Wg morphogen gradient as well.

As mentioned earlier, in the epidermis of stage 12 embryo, Wg functions as a morphogen that distributes anteriorly and forms a concentration gradient required for producing "naked cuticle" (Figure 1). Two different mechanisms were proposed by which Wg acts [25,28]. In the first model, it was proposed that the Wg gradient is established by cell spreading [28]. The cells leaving the expression domain retain inherited Wg protein in secretory vesicles and carry it forwards over a distance of up to four cell diameters. Experiments using a membrane-tethered form of Wg showed that this mechanism is sufficient to account for the normal range of Wg. In the second model, Wg can also reach distant target cells independently of protein inheritance, possibly by restricted diffusion. It is important to note that in either case retention of Wg in anterior cells is critical for producing naked cuticle cell fate. In this regard, Dly may be involved in the retention of Wg protein in these cells. If that is the case, a reduction of Dly may reduce the ability of anterior cells to retain Wg and thus fail to produce naked cuticle. Consistent with this, embryos injected with *dly* dsRNA develop cuticle that lacks denticle bands. Further detailed analysis of *dly* mutant embryos will be required to validate this model.

Transport model

Recently, Eaton and her colleagues reported an exciting finding that supports a novel cell biological mechanism for transporting Wg through a field of cells [29]. When a glycosylphosphatidyl inositol-anchored green fluorescent fusion protein was expressed in different subsets of the imaginal disc using the Gal4/UAS system, fluorescence was observed not only on the plasma membrane of expressing cells, but also in punctate structures in non-expressing cells. They have called these punctate particles "argosomes" because of their ability to travel. Several lines of evidence suggest that Wg can travel over the disc epithelium on argosomes. First, Wg-expressing cells produce argosomes from basolateral membranes that contain high levels of Wg proteins. Second, in receiving cells, Wg co-localizes with argosomes that are derived from Wg-expressing cells. Finally the rate at which argosomes spread through the disc epithelium is similar to the rate at which Wg travels.

Interestingly, following treatment with either heparinase I or III, Wg was almost undetectable on the plasma membrane of both Wg-expressing cells and the surrounding tissue. This result is consistent with our observation that extracellular Wg protein is strikingly reduced in clones of *sfl* mutant cells [11,29]. Although the production of argosomes is apparently independent of the presence of HSPGs and Wg protein, both Wg and HSPGs could be present in argosomes. It is important to note that argosomes were observed by green fluorescent protein anchored with glycosylphosphatidyl inositol linker, and that both Dally

and Dly are glycosylphosphatidyl inositol-anchored proteoglycans suggesting that these HSPGs are present in argosomes.

5. HSPGs are involved in Hh movement

Like Wnts, members of Hh family of secreted signaling molecules have also been shown to act in the specification of cell fate and patterning in many developmental processes [30]. Hh is made as a precursor protein that is autocatalytically cleaved to produce an N-terminal (Hh-N) and a C-terminal (Hh-C) fragment [30]. During this cleavage, a cholesterol moiety is covalently attached to the last amino acid of Hh-N to create biologically active processed Hh-N (Hh-Np) [30]. HhNp lacks the properties of a diffusible molecule when it is associated to a cholesterol moeity.

Our understanding of the role of HSPGs in Hh signaling mainly comes from the analysis of the *ttv* mutant phenotype [19]. As mentioned before, *ttv* encodes a *Drosophila* homolog of the human EXT-1 tumor suppressor protein that has been biochemically characterized as a HS polymerase involved in HS biosynthesis [19,20]. Analysis of *ttv* mutant clones in the wing disc led to the conclusion that HSPGs are involved in Hh movement. As shown in Figure 2, in the *Drosophila* wing disc, *hh* is expressed in the posterior compartment. Hh travels to the anterior compartment and acts at a distance of 8–10 cell diameters from the site of its production to induce the expression of its target gene *patched* (*ptc*) and *decapentaplegic* (*dpp*), and stabilizes the Cubitus Interruptus (Ci) protein along the anteroposterior boundary [30]. Homozygous mutant clones of *ttv* along this boundary show reduced *ptc* expression and Ci stabilization [19]. Importantly, Hh diffusion occurs through a *ptc* mutant clone, since ectopic induction of *ptc* expression is found in wild-type cells localized in the anterior compartment distally to a clone of *ptc* mutant cells. While Hh protein can diffuse through the *ptc* mutant clone, this does not occur through a clone of *ptc-ttv* mutant cells, arguing that Ttv is required for Hh movement [19].

Recently, a novel patched-like transmembrane protein, Dispatched (Disp), has been identified and shown to act exclusively in Hh-secreting cells to liberate HhNp from either the internal or surface membrane of the cells [31]. A current model is that Disp is required in the posterior cells to transfer cholesterol-bound Hh to the Ttv-modified cell surface HSPGs in the anterior compartment [31,32]. The Hh-HSPG complex could either transfer Hh to its receptor or could form a Hh-HSPG-Ptc ternary complex in which HSPGs may function to stabilize a Hh-Ptc complex. In this regard, the HSPGs could also be directly involved in Hh signaling. In fact, in *ttv* mutant embryos, *wg* expression decays because of defective Hh signaling. Thus, even when Hh signals to immediate neighboring cells, the HSPGs may be required for regulation of Ptc by Hh.

It is important to note that analysis of the *ttv* mutant phenotype failed to reveal a function for Ttv in either the Wg or FGFs signaling pathways suggesting that Ttv is specific to Hh signaling [20]. This is unexpected as Ttv encodes a polymerase involved in HS chain biosynthesis, such that one would predict that it should have a phenotype similar to those of either *sgl* or *sfl* mutants. Both qualitative and quantitative models have been proposed to explain this specificity. In the quantitative model, the biosynthesis of HSPG is reduced in the absence of Ttv activity but not completely eliminated. A reduced amount of HSPGs synthesized by other EXT enzymes may be sufficient for Wg and FGF signaling, but not for Hh signaling. Alternatively, in a qualitative model, specific EXTs may only modify a subset of protein cores or subset of HS to a specific protein core. HSPGs involved in Wg and FGF, but not in Hh signaling, may be modified properly in *ttv* mutant embryos. In *Drosophila*, two additional EXT family members have been identified: *brother of tout velu* (*botv*) *and sister of tout velu* (*sotv*) that encode *Drosophila EXT-3-like* and *EXT-2*, respectively (Han and Lin, unpublished results). Further analysis of the phenotype associated with these genes will clarify the function of EXT proteins in Hh and Wg signaling.

6. Conclusion

Genetic studies in *Drosophila* have revealed the critical roles of HSPGs in regulating the activity and extracellular distribution of Wg and Hh. Further detailed analysis of individual core proteins as well as enzymes involved in the modification of HS will be needed to define the precise mechanisms by which HSPG interact with extracellular factors. The combination of genetic, cell biological and biochemical approaches represents a powerful approach to characterize the function of HSPGs.

Acknowledgments

Work on HSPG in the laboratory of Norbert Perrimon (Investigator, Howard Hughes Medical Institute) is supported by the NIH and Mizutani foundation. Work on HSPG in Xinhua Lin's laboratory is supported partially by a NIH grant GM63891-01A1, a grant from Ohio Cancer Associates and a start-up fund from Cincinnati Children Hospital Research Foundation.

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