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Heparan sulfate proteoglycans are critical for the organization of the extracellular distribution of Wingless

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SUMMARY

Recent studies in *Drosophila* have shown that heparan sulfate proteoglycans (HSPGs) are required for Wingless (Wg/Wnt) signaling. In addition, genetic and phenotypic analyses have implicated the *glypican* gene *dally* in this process. Here, we report the identification of another *Drosophila glypican* gene, *dally-like* (*dly*) and show that it is also involved in Wg signaling. Inhibition of *dly* gene activity implicates a function for DLY in Wg reception and we show that overexpression of DLY leads to an accumulation of extracellular Wg. We propose that DLY plays a role in the extracellular distribution of Wg. Consistent with this model, a dramatic decrease of extracellular Wg was detected in clones of cells that are deficient in proper glycosaminoglycan biosynthesis. We conclude that HSPGs play an important role in organizing the extracellular distribution of Wg.

Key words: HSPG, Wg signaling, Morphogen, *Drosophila melanogaster*

INTRODUCTION

Heparan sulfate proteoglycans (HSPGs) are abundant cell-surface molecules that are part of the extracellular matrix. HSPGs consist of a protein core (such as syndecan and glypicans) to which heparan sulfate glycosaminoglycan (HS GAG) chains, which are long unbranched chains of sulfated repeating disaccharides, are attached (Kjellén and Lindahl, 1991; Lindahl et al., 1998). Numerous biochemical and cell-culture assays have suggested that HSPGs are involved in a variety of biological phenomena such as organogenesis, embryonic development, angiogenesis, regulation of blood coagulation, cell adhesion and lipid metabolism (Lindahl et al., 1994; Salmivirta et al., 1996; Rosenberg et al., 1997). In the context of signal transduction, HSPGs have been implicated in a number of signaling pathways, in particular those of Fibroblast growth factor (FGF), Wnt, Transforming growth factor β, (TGFβ) and Hedgehog (Hh; Reichsman et al., 1996; Lee et al., 1994; Rapraeger et al., 1991; Ruppert et al., 1996; The et al., 1999).

The function of HSPGs in signaling is well understood in the context of FGF signaling where they have been shown to stabilize the interaction between FGF and their transmembrane receptor protein tyrosine kinases (FGFR). In the FGF context, the HSPGs act as abundant low affinity receptors, and recent structural studies have provided evidence that the HSPGs play a role in the interaction between FGFs and FGFRs (Plotnikov et al., 1999). Importantly, these biochemical and structural findings are consistent with in vivo studies. In particular, genetic studies in *Drosophila* have identified mutations in two genes required for HS GAG biosynthesis; *sugarless* (*sgl*; UDP-glucose dehydrogenase; Häcker et al., 1997; Binari et al., 1997; Haerry et al., 1997) and *sulfateless* (*sfl*; *N*-deacetylase/*N*-sulphotransferase; Lin and Perrimon, 1999). Embryos that develop in the absence of either *sgl* or *sfl* gene activity are defective in FGF signaling (Lin et al., 1999).

Recently, evidence has been obtained in *Drosophila* that HSPGs are involved in the movement of the heparin-binding Hh proteins through tissues (Bellaiche et al., 1998; The et al., 1999). In the wing imaginal disc, Hh travels 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*) along the anteroposterior (AP) boundary (Strigini and Cohen, 1997; Mullor et al., 1997). Results from mosaic analyses of *tout-velu* (*ttv*) mutations (Bellaiche et al., 1998), have revealed that *ttv* gene activity is required in Hh-receiving cells for the movement of Hh. Because, Ttv encodes a type II transmembrane HS polymerase enzyme, it has been proposed that a Ttv-modified HSPG is required for the proper distribution of the membrane-targeted cholesterol-modified Hh (HhNp) molecule through tissue (The et al., 1999). 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 (Burke et al., 1999). One current model is that the Ttv-modified cell

surface HSPG interacts with HhNp to release it from Disp, thus allowing HhNp to move through tissues (Burke et al., 1999; Ingham, 2000).

Other evidence has also implicated HSPGs in Wingless (Wg) signaling. In tissue culture cells, Wg proteins are tightly associated with cell membranes and the extracellular matrix, possibly through naturally occurring sulfated proteoglycans (Reichsman et al., 1996). Further, mutations in *sgl* and *sfl* are defective in Wg signaling (Häcker et al., 1997; Binari et al., 1997; Haerry et al., 1997; Lin and Perrimon, 1999). Recently, the *Drosophila* gene *dally*, has been proposed to encode the protein core of the HSPGs involved in Wg signaling (Lin and Perrimon, 1999; Tsuda et al., 1999) and it is known that *dally* encodes a glycosyl-phosphatidyl inositol (GPI)-linked glypican molecule. Loss of *dally* gene activity is associated with defects reminiscent of loss of *wg* activity, and *dally* and components of the Wg signaling pathway interact genetically. These data support the model that Dally plays a role in the reception of the Wg signal.

During patterning of the imaginal discs, Wg acts both as a short- and a long-range inducer (Zecca et al., 1996; Neumann and Cohen, 1997). In patterning the wing blade, Wg is expressed in a narrow stripe of cells at the dorsoventral (DV) compartment border and acts in a long-range manner. Wg acts up to 20-30 cell diameters away from its site of synthesis and triggers a graded transcriptional response of target genes, such as *distalless* (*dll*), which is a feature of morphogen molecules (Zecca et al., 1996; Neumann and Cohen, 1997). Recently, Strigini and Cohen (Strigini and Cohen, 2000) were able to visualize the Wg extracellular protein gradient, thus providing direct support for this model. The transmembrane Wg receptor encoded by *Drosophila frizzled 2* (*Dfz2*; Bhanot et al., 1996) has been shown to play a critical role in shaping the distribution of Wg. Dfz2 is required to post-transcriptionally stabilize Wg, and thus allows it to reach cells far from its site of synthesis (Cadigan et al., 1998).

Two models have been proposed with regards to the function of HSPGs in Wg signaling (Reichsman et al., 1996). As observed in the case of FGF, HSPGs may be required for stabilizing a complex between Wg and its receptor. Alternatively, as in the case of heparin-binding growth factors such as vascular endothelial growth factor, heparin-binding epidermal growth factor (EGF) and TGFβ, the HSPGs may restrict the extracellular diffusion of the ligand.

We have examined the function of HSPGs in Wg signaling. First, we describe a novel glypican molecule in *Drosophila* that we have named Dally-Like (DLY) and show that it is required for Wg signaling. Interestingly, overexpression of DLY leads to an accumulation of extracellular Wg, suggesting that DLY plays a role in Wg extracellular distribution. Finally, we found that *sfl* mutant cells do not trap extracellular Wg proteins. Taken together, our results suggest that HSPGs play an important role in the distribution of extracellular Wg.

MATERIALS AND METHODS

Molecular cloning of *dly*

A 0- to 4-hour embryonic cDNA library (Brown and Kafatos, 1988) was screened using a ^{32}P-labeled 0.7 kb *Bam*HI fragment from the EST clone CK00242 (Kopczynski et al., 1998) as a probe. A positive clone carrying the entire coding region was subcloned into pBluescript II KS (pBS(KS)-dly, *Hin*dIII-*Eco*RI) and sequenced using synthetic oligonuleotides primers.

UAS construct and ectopic expression

UAS-dlyWT was created by cloning the full-length (*Xho*I-*Xba*I) *dly* fragment from pBS (KS)-dly into pUAST. The construct was introduced into w^{1118} host by P-mediated germline transformation (Rubin and Spradling, 1982). Targeted ectopic expression was accomplished using the UAS/Gal4 system (Brand and Perrimon, 1993).

The Gal4 drivers and UAS lines used in this study were: *UAS-wg* (Binari et al., 1997), $UAS\text{-}arm^{act}$ (Pai et el., 1997), *C96-Gal4* (Gustafson and Boulianne, 1996), *en-Gal4* on the second chromosome and *ptc-Gal4* on the third chromosome.

In situ hybridization and RNA-mediated interference

dly mRNA was detected in whole-mount embryos using digoxigenin-UTP-labeled RNA probes prepared from the pBS(KS)-dly.

The CK00242 plasmid containing a 1.2 kb fragment of the C terminus *dly* coding sequence and pBS(KS)-dally containing a 1.1 kb *Eco*RI-*Bam*HI fragment from LP 11764, which includes the entire coding region of *dally*, were linearized with the appropriate restriction enzymes, and transcribed in vitro with Ambion T3 and T7 Megascript Kits following the manufacturers instructions. Transcripts were annealed in TE buffer (10 mM Tris-HCl, 8.0 and 1 mM EDTA, 8.0) after heating to 100°C for 1 minute and cooling to room temperature overnight. Annealed transcripts were analyzed on 1% agarose gels to confirm the size of the annealed dsRNA. Wild-type precellular embryos were injected as described by Kennerdell and Carthew (Kennerdell and Carthew, 1998) with dsRNA: either 3 μM *dly* dsRNA, 3 μM *dally* dsRNA or 1.5 μM each of *dly* and *dally* dsRNAs. Embryos were incubated at 18°C under oil for 2 days. For cuticle preparation, embryos were mounted in Hoyer's medium/lactic acid.

Antibody labeling

Cytoplasmic Wg proteins were detected as described by Cadigan and Nusse (Cadigan and Nusse, 1996). For labeling extracellular Wg proteins, third instar larvae were dissected in ice-cold Schneider's M3 medium (Sigma) and incubated with mouse anti-Wg antibody (1:3) for 1 hour on ice. After washing three times with cold PBS, larvae were fixed in PBS containing 4% formaldehyde at room temperature for 20 minutes. Larvae were rinsed three times with PBS for 40 minutes and incubated with fluorescent secondary antibody overnight (Strigini and Cohen, 2000). Other antibodies used were: mouse anti-Dll (1:10) and mouse anti-Ac (1:10).

Generation of anti-DLY antibodies and western blot analysis

Rabbit polyclonal antibodies were raised against the following peptides QQRRKQQNNRDDNDD. For the purification of the antiserum, a CNBr-activated Sepharose 4B column was used. Wild-type pupae or $sfl^{l(3)03844}/sfl^{9B4}$ pupae were homogenized in SDS loading buffer and heated to 100°C for 5 minutes. Immunoblotting was performed with the Dly antibodies (1:100).

Loss-of-function clonal analysis

To study the role of HSPGs in extracellular distribution of Wg, *y w hsflp; ubiquitin-GFP FRT2A/TM6C* females were crossed with $sfl^{l(3)03844}$ *FRT2A/TM6C*. Wing discs from Non-Tubby larvae were dissected and stained. For the generation of adult *sfl* somatic clones in the wing, females of *y w hsflp122;* $sfl^{l(3)03844}$ *FRT2A/TM3, Sb* were crossed with males of *y w; P[y$^+$] FRT2A/TM3, Sb*. Larvae from this cross were heat-shocked for 2 hours during the first or second instar larval stage. Adult wings were mounted in Euparal for observation.

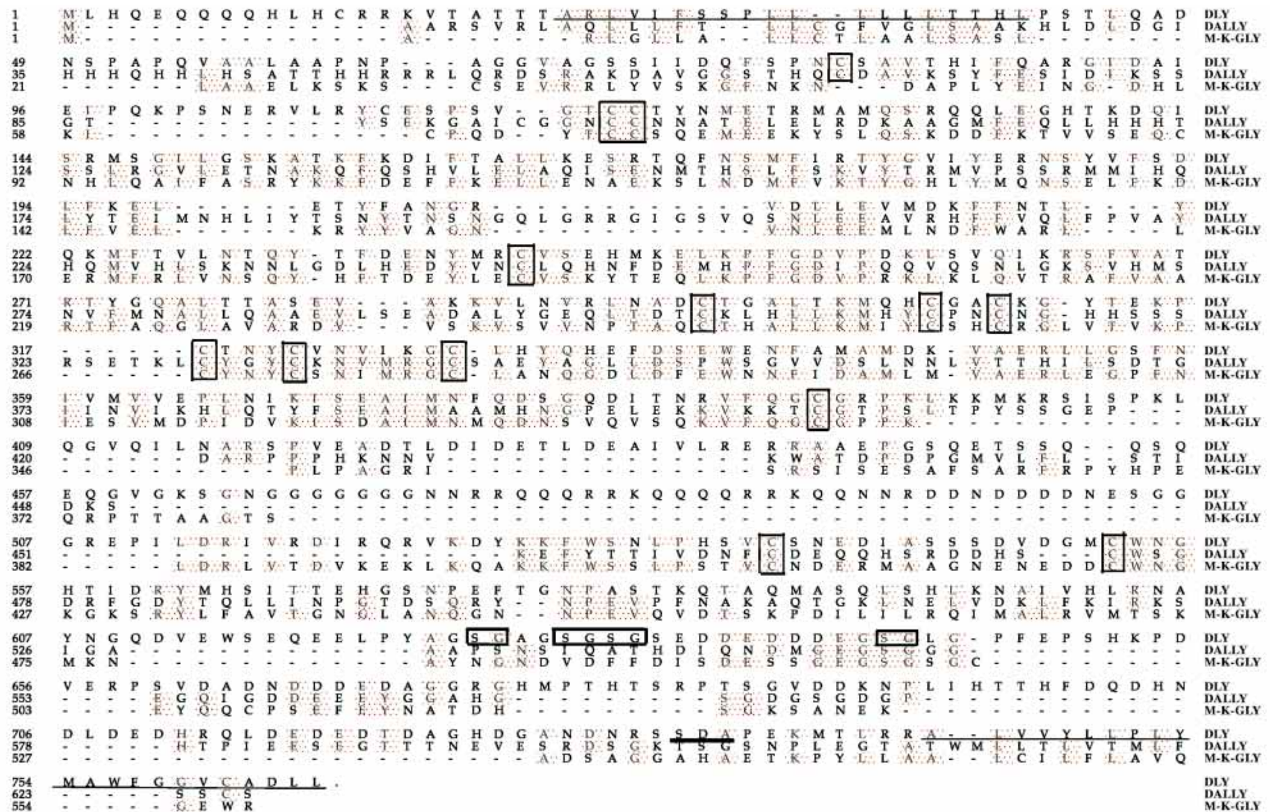

Fig. 1. The amino acid sequence of DLY. The entire predicted amino acid sequence of DLY is aligned with Dally and mouse K-Glypican. DLY is 22% and 35% identical to Dally and mouse K-Glypican, respectively. Identical residues are stippled in red. The hydrophobic stretches for the predicted signal sequences involved in secretion (amino acid residues 23-41), and GPI-anchoring (residues 744-765) are underlined. The predicted GPI-anchor attachment sites are indicated by bold underlining. The position of cysteine residues in glypican family members and serine-glycine dipeptide sequences in DLY are boxed.

RESULTS

Molecular cloning of a novel *Drosophila glypican* gene

Previously, studies have implicated one glypican molecule, Dally, in Wg signaling (Tsuda et al., 1999; Lin and Perrimon, 1999). However, because numerous *glypican* genes are present in other animals (Bernfield et al., 1999; Perrimon and Bernfield, 2000; Lander and Selleck, 2000), we searched the *Drosophila* database for additional *glypican* family members. We found one EST clone that showed some sequence similarity to *dally* and subsequently cloned a full length cDNA (see Materials and Methods). The sequence of the cDNA revealed a potential open reading frame of 765 amino acid residues (Fig. 1), with 22% and 35% identity to Dally and mouse K-glypican, respectively (Fig. 1). The predicted primary structure of the molecule exhibits the hallmarks of a glypican protein. The hydrophilicity plot of the new molecule is similar to those of the other members of the glypican family, which is characterized by the presence of NH2- and COOH-terminal hydrophobic signal sequences (data not shown). In addition, this molecule possesses four consensus serine/glycine dipeptide sequences for GAG attachment sites, and a signal sequence for a GPI-moiety attachment site at the COOH-terminal region (Fig. 1). Moreover, the number and position of cysteine residues, which are a unique feature of glypican family members, are almost completely conserved in the predicted protein (Fig. 1). These results indicate that we have identified a novel *Drosophila* member of the glypican family, and we have named it Dally-Like (DLY). Hybridization using a *dly*-specific probe to polytene chromosomes from salivary glands localized the *dly* gene to the cytological division 70F on the third chromosome (data not shown). Finally, northern blot analysis revealed that *dly* encodes a single major 3.8 kb transcript (data not shown).

dly is a novel segment polarity gene

To determine the function of DLY, we first determined its expression in embryos by in situ hybridization. *dly* transcripts are uniformly expressed at early embryonic stages (Fig. 2A), but by stage 8 they are enriched in stripes (Fig. 2B). Double staining for *dly* mRNA and Wg protein show that *dly* transcripts are preferentially expressed in three to four cells anterior to the *wg*-expressing cells (data not shown). Interestingly, this expression pattern is similar to that of both *dally* and *Dfz2* (Bhanot et al., 1996; Tsuda et al., 1999; Lin and Perrimon, 1999).

Next, in an attempt to assess the function of DLY during embryogenesis, we used the RNA interference (RNAi) method (Kennerdell and Carthew, 1998) to perturb DLY protein synthesis. Embryos were injected with a *dly* double-stranded RNA (dsRNA) (see Materials and Methods). These embryos, referred to as *dly* dsRNA embryos in the text, showed the

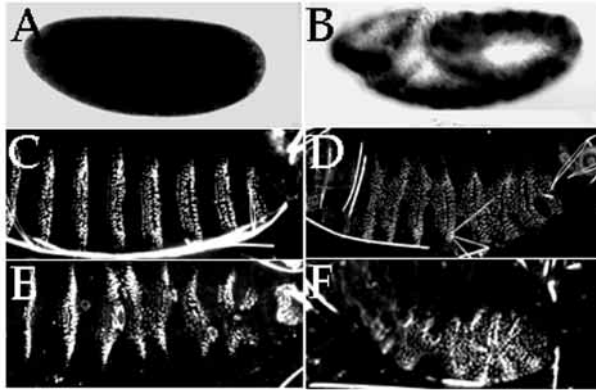

Fig. 2. *dly* is a novel segment polarity gene involved in Wg signaling. In situ hybridization of wild-type embryos using a *dly*-specific probe reveals that (A) *dly* RNA is uniformly expressed at stage 2, and (B) enriched in a segmentally repeated pattern at stage 8. (D) Embryos injected with a *dly* dsRNA (3 μM) show a segment polarity phenotype characterized by the absence of naked cuticle (compare with the embryos injected with buffer in C). (E) Similarly, embryos injected with *dally* dsRNA (3 μM) develop *wg*-like cuticle defects. (F) Embryos injected with an equimolar mixture of *dly* and *dally* dsRNAs (1.5 μM each of *dly* and *dally* dsRNAs) exhibit more severe segment polarity phenotype.

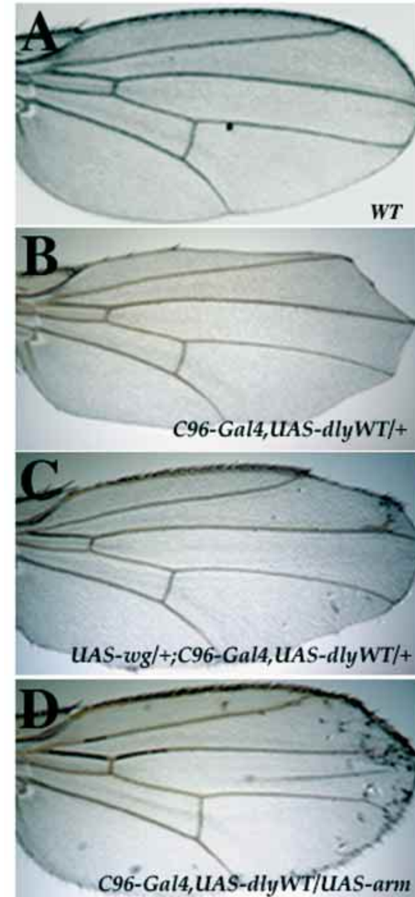

Fig. 3. Ectopic expression of *dly* induces a *wg*-like phenotype. Ectopic expression of *dly* using the *C96-Gal4* driver generates wing margin defects and loss of sensory bristles. (B) A *C96-Gal4/UAS-dlyWT* wing; compare with the wild type shown in A. (C,D) Co-expression of *wg* (C; *UAS-wg/+; C96-Gal4,UAS-dlyWT/+*), or a gain-of-function Arm (D; *C96-Gal4,UAS-dlyWT/UAS-arm^{act}*) are sufficient to rescue both the wing notching phenotype and the loss of sensory bristles associated with overexpression of DLY. In addition, ectopic margin bristles close to the wing margin are observed.

absence of naked cuticle (Fig. 2D). This phenotype is reminiscent of loss of either *wg* or *hh* gene activities. The segment polarity phenotype is also found when the activity of *dally*, which is required for Wg signaling in the embryo (Lin and Perrimon, 1999; Tsuda et al., 1999), was disrupted by RNAi, though the effect is less severe in *dally* dsRNA embryos than in *dly* dsRNA embryos (Fig. 2E). However, when compared with embryos injected with either *dly* dsRNA or *dally* dsRNA alone, embryos injected with an equimolar mixture of *dly* and *dally* dsRNAs showed more severe segment polarity phenotypes (Fig. 2F). These embryos are smaller, particularly in the tail region. They also show an entire transformation of naked cuticle into cuticle with denticles, which is observed in *wg* or *hh* null mutations. Because *dally* does not appear to play a role in Hh signaling (Lin and Perrimon, 1999; Tsuda et al., 1999), the interaction between *dally* and *dly* observed in the RNAi interference experiment suggests that DLY and Dally function synergistically in Wg signaling (Fig. 2F). Altogether, our results suggest that *dly* is a novel segment polarity gene that potentiates Wg signaling.

Ectopic expression of DLY induces loss of Wg signaling

To further examine the role of DLY in Wg signaling, we analyzed the function of DLY during wing imaginal disc development. *dly* transcripts are uniformly expressed in wing discs (data not shown). In the third instar imaginal disc, *wg* is expressed at the DV compartment border and acts over short and long ranges to pattern the wing disc. Short range Wg signaling induces the expression of the proneural gene *achaete* (*ac*) in a stripe on each side of the DV boundary, while long range Wg signaling controls the expression of *Distal-less* (*Dll*) within the wing blade (Zecca et al., 1996; Neumann and Cohen, 1997). We reasoned that overexpression of DLY might activate Wg signaling because *dly* dsRNA-injected embryos resemble those that have lost Wg activity. Interestingly, overexpression of *dly*, using the *C96-Gal4* driver, that is highly expressed at the DV boundary of the wing disc, resulted in severe wing margin defects and loss of sensory bristles (Fig. 3B). These phenotypes are reminiscent of the phenotypes seen when Wg activity is reduced in the wing (Couso et al., 1994). Consistent with the adult wing phenotype, Ac expression is dramatically decreased in wing discs overexpressing DLY (Fig. 4B). Furthermore, when *dly* is overexpressed using the *engrailed-Gal4* (*en-Gal4*) driver, the expression of Dll is reduced in the posterior compartment (Fig. 4D).

Next, we tested whether overexpression of transducers of the Wg signal can rescue the loss-of-function *wg*-like phenotypes associated with *dly* overexpression. Ectopic expression of either Wg or a gain-of-function Armadillo (Arm) can rescue the wing margin defects, and induced ectopic bristles that are characteristic of ectopic expression of the Wg pathway (Axelrod et al., 1996; Zhang and Carthew, 1998) (Fig. 3C,D). Altogether, these results suggest that overexpression of DLY

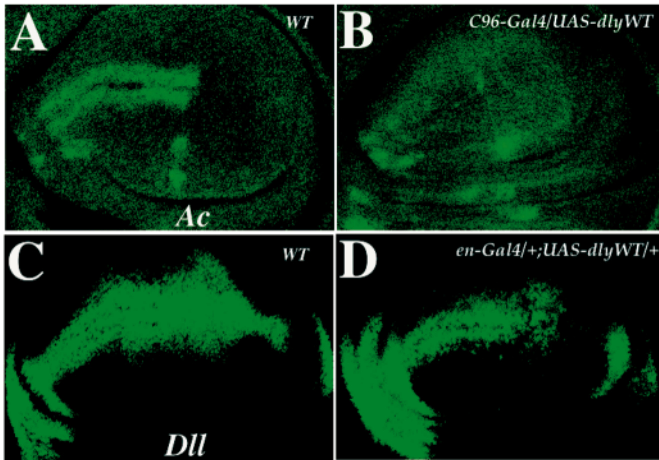

Fig. 4. Effect of overexpressed DLY on Wg short- and long-range target genes. (A) *Ac* is expressed in two parallel bands at the presumptive anterior wing margin, and is a short range target gene of Wg (Zecca et al., 1996; Neumann and Cohen, 1997b). (B) In a *C96-Gal4/UAS-dlyWT* wing disc, *ac* expression is dramatically decreased. (C) In the wing blade, *Dll*, a long-range target gene of Wg, is normally expressed in a wide domain with its highest level at the DV boundary. (D) In a *en-Gal4/+; UAS-dlyWT/+* imaginal discs, the expression of *Dll* is abolished in the posterior compartment (compare to the expression in the anterior compartment).

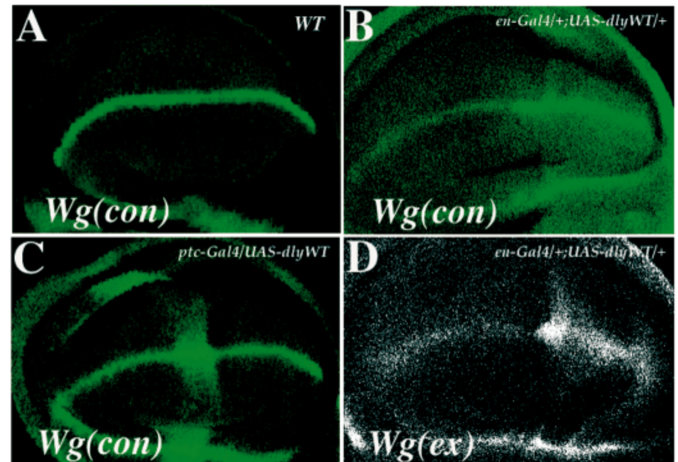

Fig. 5. Over-expressed DLY sequesters extracellular Wg. Wg (in green) is detected in a narrow stripe of 3 to 4 cells straddling the DV boundary of the wing disc using a conventional (con) staining protocol that detects mostly cytoplasmic Wg (A-C; see Results). (A) Ectopic expression of DLY-WT, using *en-Gal4* and *ptc-Gal4,* results in an increased accumulation of Wg. (B) Trapping of Wg in the posterior compartment in an *en-Gal4/+; UAS-dlyWT/+* disc, and (C) trapping of Wg at the anterior/posterior (A/P) boundary in a *ptc-Gal4/UAS-dlyWT* disc. (D) Imaginal discs were incubated with anti-Wg antibody prior to fixation to visualize extracellular (ex) Wg. Over-expression of DLY is associated with accumulation of extracellular Wg (white) to high level in the entire posterior compartment.

blocks Wg signaling in the wing disc, and that of DLY acts upstream of Arm.

Overexpression of DLY sequesters Wg

Since DLY is an extracellular GPI-linked molecule, we reasoned that patterning defects associated with DLY overexpression might reflect the ability of DLY to sequester Wg, and thus prevent it from accessing and activating Dfz2. To visualize the effect of overexpressed DLY on Wg distribution, we overexpressed DLY using various Gal4 lines and stained the wing discs with anti-Wg monoclonal antibodies. We used two different staining protocols to detect Wg distribution. The first one, involves fixing the tissue before staining, and thus detects mostly cytoplasmic Wg present in either secretory or internalized vesicles (van den Heuvel et al., 1989; Neumann and Cohen, 1997). In the second protocol, the tissue is incubated with the antibody prior to fixation and mostly detects extracellular Wg (Strigini and Cohen, 2000).

Using the first protocol, in wild-type discs, Wg protein is found at high levels in a narrow stripe of three to five cells straddling the DV boundary (Fig. 5A). Following overexpression of DLY using either *en-Gal4* or *ptc-Gal4* a striking increased accumulation of Wg protein is observed (Fig. 5B,C). Using the second protocol, extracellular Wg is organized in a gradient at the basolateral surface of *wg*-expressing and nearby cells. Following overexpression of DLY, we detected an increased accumulation of extracellular Wg protein. This result indicates that DLY can affect extracellular Wg distribution (Fig. 5D).

HSPGs are required to increase the local concentration of Wg ligand for its receptors

Considering the roles of Dally (Tsuda et al., 1999; Lin and Perrimon, 1999) and DLY, there are at least two HSPGs at the cell surface of wing disc cells involved in Wg signaling. To generate mutant cells that lack all GAGs and determine the role of the HSPGs in Wg signaling, we generated mutant clones of cells that do not properly synthesize GAGs. *sgl* mutations cannot be used for this analysis because *sgl* acts in a cell non-autonomous manner (data not shown), presumably because the enzyme synthesizes glucuronic acid that diffuses between cells. However, *sfl* is involved in GAG modification and in its absence, the GAG chains are not synthesized properly. To determine whether the GAG chains of DLY are modified by *sfl*, we examined DLY proteins expressed in wild-type or *sfl* mutant pupae, by western blot analysis using anti-DLY antibodies (Fig. 6). The predicted size for DLY is 80 kDa, and in wild-type pupae, DLY protein appeared as a broad band that migrates to around 80-110 kDa, which presumably results from addition of GAG chains onto the core protein. In *sfl* mutant pupae, the modified form of DLY protein is significantly reduced and the sharp band of the core protein is increased. This results indicate that *sfl* plays a role in DLY modification.

Using the staining protocol that detects cytoplasmic Wg, we could not detect an alteration in the expression of intracellular Wg in *sfl* mutant clones, indicating that *sfl* mutant cells normally transcribe *wg* and do not accumulate Wg (Fig. 7A,B). However, using the extracellular staining method, a dramatic decrease of extracellular Wg was detected in *sfl* mutant cells (Fig. 7C,D). Extracellular Wg has been shown to be mainly associated with the basolateral surface of cells, and GPI-anchored protein are thought to be primarily attached to the basal part of the cells. Together, these results suggest that the HSPGs are involved in extracellular Wg accumulation.

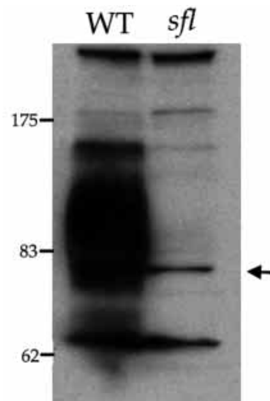

Fig. 6. GAG modification of DLY requires *sfl* activity. Total proteins from pupae were analysed by 6% SDS-PAGE followed by western blotting with anti-DLY antibodies. In wild-type pupae (left lane), DLY migrates as a smear around 80-110 kDa, characteristic of heparan sulfate-modified DLY. In $sfl^{l(3)03844}/sfl^{9B4}$ pupae (right lane), only a single band of the unmodified form is detected around 80 kDa. Arrow indicates the core protein.

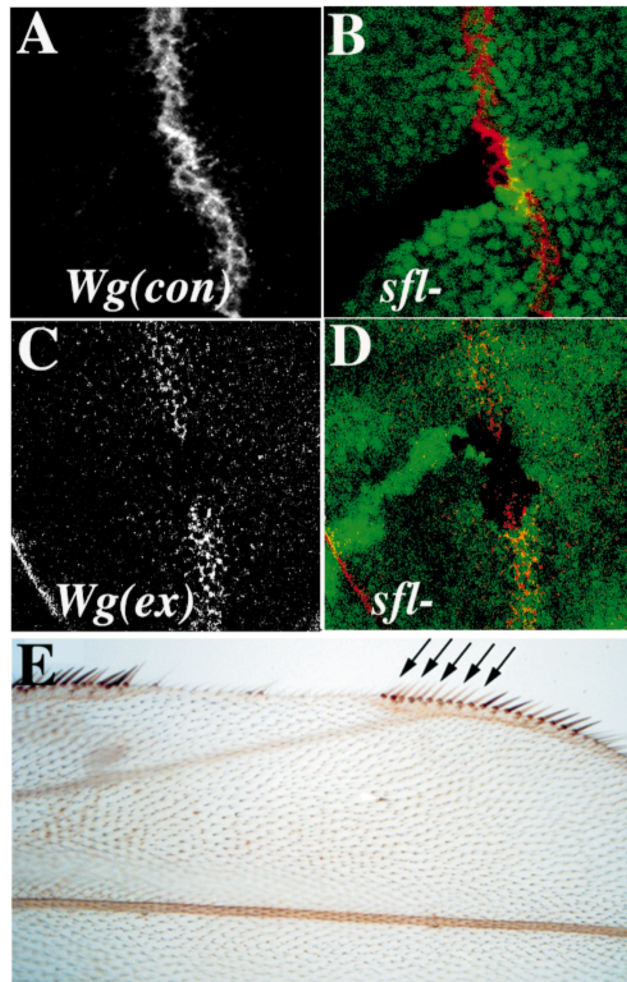

Fig. 7. HSPGs and Wg extracellular distribution. Cytoplasmic (A,B) and extracellular Wg protein (C,D) were visualized using different staining methods. (A,C) Wg expression is shown separately. (B,D) *sfl* mutant clones were detected by the absence of GFP (see Materials and Methods). No alteration in the expression of intracellular Wg proteins (white, red) in *sfl* mutant clone was observed, suggesting that *sfl* cells transcribe and secrete Wg normally (A,B). A dramatic decrease of extracellular Wg (white, red) was detected in *sfl* mutant cells (C,D). Clones of *sfl* mutant in adult wing are associated with wing margin nicks. (E) However, near wild-type cells, *sfl* mutant cells (marked with *yellow*, arrows) differentiate properly. The presence of rescued *yellow sfl* bristles reveal the local non-autonomy of *sfl*.

Importantly, high accumulation of extracellular Wg can be detected on *sfl* mutant cells located adjacent to wild-type cells (Fig. 7D), suggesting that HSPGs act locally in a cell non-autonomous manner. Consistent with this observation, adult wing patterning in *sfl* mutant clones show local cell non-autonomy as well. Clones of *sfl* mutant cells are associated with wing margin defects, suggesting that *sfl* is required for Wg signaling, however some of the *sfl* mutant cells located near wild-type margin cells have a wild-type morphology (Fig. 7E).

Taken together, our results suggest that HSPGs are involved in restricting Wg diffusion. Further, the local cell non-autonomy observed in *sfl* mutant clones may indicate that HSPGs may not be absolutely required for the binding of Wg to its transducing receptor(s) (see Discussion).

DISCUSSION

Wnt proteins are one of the first examples of secreted molecules that have been shown to be associated with both short- and long-range signaling activities during animal development (McMahon and Moon, 1989; Struhl and Basler et al., 1993). In the *Drosophila* wing imaginal disc, Wg is secreted from a narrow stripe, 3-5 cells wide along the DV boundary and diffuses symmetrically in the extracellular space. Wg regulates the expression of different target genes in a concentration-dependent manner (Zecca et al., 1996; Cadigan et al., 1998). Recently, Strigini and Cohen (2000) have shown that there is a basally located gradient of extracellular Wg, which is most likely associated with extracellular matrix.

Here, we have identified and characterized a novel component of the Wg signal transduction pathway. We provide evidence that the putative GPI-attached Dally-Like (DLY) protein potentiates Wg signaling pathway during both embryonic and imaginal disc development. Our results demonstrate that overexpression of DLY in wing discs sequesters Wg and disrupts its distribution. Finally, we provide direct in vivo evidence that HSPGs play a role in increasing the local concentration of extracellular Wg protein.

DLY encodes a HSPG core protein involved in Wg signaling

The predicted amino acid sequence of full-length DLY reveals that it is a new member of the glypican family of proteins (Fig. 1). These proteins possess a C-terminal hydrophobic region that is thought to be required for processing and attachment to the external leaflet of the plasma membrane through a GPI linkage. Recent studies by Strigini and Cohen (Strigini and Cohen, 2000) have clearly detected a broad gradient of extracellular Wg protein that is concentrated exclusively on the basolateral surface of *wg*-expressing and nearby cells. This distribution of Wg is consistent with an involvement of glypican proteins in Wg signaling because it has been reported that most of these molecules are located to the basolateral surface of polarized epithelial cells (Mertens et al., 1996).

We have obtained evidence that implicates DLY in Wg signaling. First, *dly* transcripts are expressed, as is the Wg receptor Dfz2, at higher levels in segmentally repeated stripes anterior to the *wg*-expressing cells during segmentation (Fig. 2B). Second, RNA-mediated interference of *dly* supports the model that DLY potentiates Wg signaling, since *dly* dsRNA embryos exhibit a *wg*-like segment polarity phenotype (Fig. 2D). Third, double mutant embryos of *dly* and *dally* from RNA interference experiments showed an interaction between DLY and Dally (Fig. 2F). Fourth, overexpression of DLY in the imaginal tissue generates a *wg*-like phenotype (Fig. 3B) and is associated with trapping of extracellular Wg (Fig. 5D). Finally, the phenotype associated with overexpression of DLY can be rescued by co-expression of a gain-of-function form of Arm (Fig. 3D), which is consistent with the model that overexpressed DLY antagonizes Wg signaling.

Transducing Wg receptors and HSPGs

Morphogens are defined as localized factors that can diffuse and directly specify different cellular identities among a group of cells in a concentration-dependent manner (Wolpert, 1989). In the wing imaginal discs, Wg acts as a morphogen because it is organized in an extracellular protein gradient and activates the expression of target genes such as *ac, Dll* and *vestigial* (*vg*), in a dose-dependent manner (Zecca et al., 1996; Neumann and Cohen, 1997). In theory, proteins that bind the morphogen molecules can play a role in shaping its extracellular distribution. Indeed, in the case of Wg, it has been shown that *Dfz2* is downregulated by Wg signaling. Thus, it has been proposed that a graded distribution of Dfz2 protein, which is opposite to the Wg gradient, exists in the wing disc (Cadigan et al., 1998).

Wg binds tightly to GAGs (Reichsman et al., 1996) and appears to interact with DLY, raising the possibility that DLY is also involved in shaping the gradient of extracellular Wg. We have observed a high level of Wg accumulation in wing discs overexpressing *dly*, suggesting that DLY may have a high capacity to bind Wg in vivo. Thus, the loss of Wg signaling activity that results from ectopic expression of DLY may simply be due to Wg being trapped by the HSPG and unavailable for binding to Dfz2. This model suggests that the amount of HSPG and Dfz2 transducing receptor may have to be precisely monitored to ensure proper patterning.

For simplicity in our discussion we have only taken DLY and Dfz2 into account. However, the situation is more complex as there are at least two other putative Wg receptors, Fz1 and DFz3 (Sato et al., 1999), as well as one additional glypican molecule Dally (Lin and Perrimon, 1999; Tsuda et al., 1999), present at the surface of the wing disc cells. In the embryo, Fz1 is able to substitute for Dfz2 in transducing Wg (Kennerdell and Carthew, 1998; Chen and Struhl, 1999; Bhanot et al., 1999). DFz3, however, acts as an antagonist of the pathway. Furthermore, previous studies have shown a positive requirement for Dally in Wg signaling. With regards to Dally, we have not been able to detect a striking effect on Wg distribution associated with its overexpression in the imaginal disc (data not shown). Possibly, this may reflect that Dally, unlike DLY, has a relatively low capacity to bind Wg in vivo (see also Strigini and Cohen, 2000). Future analyses of the mechanism of Wg signaling and distribution in the wing discs will have to take these other molecules into account. Furthermore, it will be important to monitor precisely the expression of each molecule in both wild-type and mutant contexts, as intricate regulatory loops exist. For example, *DFz3* is positively transcriptionally regulated, while both *Dally* and *Dfz2* are negatively regulated by Wg signaling (Cadigan et al., 1998; X. L. and N. P., unpublished).

The role of HSPGs in Wg signaling

To determine the role of HSPGs on Wg distribution, we generated clones of *sfl* mutant cells. In the absence of Sfl activity, the GAG chains of all HSPGs are either not synthesized or modified properly (Lin and Perrimon, 1999; Tsuda et al., 1999).

sfl mutant cells transcribe *wg* normally because we detected intracellular Wg protein as in the wild type. Furthermore, there is no effect on Wg secretion in these cells since we could not detect Wg accumulation, as observed in *porcupine* or *shibire* mutant clones (Strigini and Cohen, 2000). However, we could detect a dramatic decrease of extracellular Wg protein in *sfl* mutant clones, suggesting that the trapping of Wg to *sfl* mutant cells is impaired.

Our mosaic analysis of *sfl* reveals a local cell non-autonomy of the HSPG (Fig. 7D,F), suggesting that the HSPG may not be important for activation of signaling receptors as previously described for the role of those molecules in FGF signaling (see Introduction). This result is in accordance with our previous observations that overexpression of Wg protein can bypass the requirement of HS GAG in Wg signaling (Häcker et al., 1997). Altogether, our findings are consistent with the idea that the function of the HSPG in Wg signaling is to limit Wg extracellular diffusion. Thus when DLY is overexpressed, Wg is trapped and cannot be presented to its transducing receptors. One possible model is that in the wild type, the abundant GAG chains of HSPG are required for reducing dimensionality for ligands, which in turn allow more frequent encounters with the high affinity signaling receptor molecules, which are in low abundance. The local non-autonomy associated with *sfl* mutant clones could then be explained by the local diffusion of Wg from the surface HSPG present on nearby wild-type cells. An alternative model, which is not mutually exclusive with the previous one, is that the HSPG prevent Wg from being degraded by extracellular proteases. Thus, in the *sfl* mutant clone, extracellular Wg may not be detected because Wg is rapidly degraded. In conclusion, although the detail mechanism of action of the HSPG in Wg signaling is not yet fully understood, our results indicate that HSPGs play an important role in the distribution of extracellular Wg.

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Note added in proof

The DNA sequence of *dally-like* has been submitted to GenBank and has been assigned the Accession Number AF317090.

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