Drosophila glypicans control the cell-to-cell movement of Hedgehog by a dynamin-independent process

Chun Han, Tatyana Y. Belenkaya*, Bei Wang* and Xinhua Lin†

Division of Developmental Biology, Children's Hospital Medical Center, Cincinnati, OH 45229, USA

*These authors contributed equally to this work
†Author for correspondence (e-mail: linxy@chmcc.org)

Accepted 30 October 2003

Summary

The signalling molecule Hedgehog (Hh) functions as a morphogen to pattern a field of cells in animal development. Previous studies in Drosophila have demonstrated that Tout-velu (Ttv), a heparan sulphate polymerase, is required for Hh movement across receiving cells. However, the molecular mechanism of Ttv-mediated Hh movement is poorly defined. We show that Dally and Dally-like (Dly), two Drosophila glypican members of the heparan sulphate proteoglycan (HSPG) family, are the substrates of Ttv and are essential for Hh movement. We show that embryos lacking dly activity exhibit defects in Hh distribution and its subsequent signalling. However, both Dally and Dly are involved and are functionally redundant in Hh movement during wing development. We further demonstrate that Hh movement in its receiving cells is regulated by a cell-to-cell mechanism that is independent of dynamin-mediated endocytosis. We propose that glypicans transfer Hh along the cell membrane to pattern a field of cells.

Key words: Hedgehog, Heparan sulphate proteoglycans, Glypican, Dally; Dally-like, Drosophila, Morphogen gradient formation, Signaling

Introduction

Members of the Hedgehog (Hh) family of secreted signalling proteins function as organizers in controlling cell growth and patterning during animal development (Ingham and McMahon, 2001). Hh proteins have been shown to function as morphogens that signal over a distance to directly induce the expression of target genes in a concentration dependent manner (Briscoe et al., 2001; Mullor et al., 1997; Strigini and Cohen, 1997; Struhl et al., 1997; Zecca et al., 1995). The mature forms of all Hh members are dually lipid modified (Ingham and McMahon, 2001; Jeong and McMahon, 2002). The precursor of Hh is first autocatalytically cleaved to produce an N-terminal (Hh-N) and a C-terminal (Hh-C) fragment (Lee et al., 1994; Porter et al., 1996b). A cholesterol moiety is covalently attached to the last amino acid of the Hh-N to create Hh-Np (p stands for processed) that is responsible for the biological activities of all Hh proteins (Ingham and McMahon, 2001; Lee et al., 1994; Porter et al., 1996b). The N-terminal region of the cholesterol modified Hh is further modified by addition of palmitate that is essential for its signalling activity (Amanai and Jiang, 2001; Chamoun et al., 2001; Lee and Treisman, 2001; Micchelli et al., 2002; Pepinsky et al., 1998). Lipid-modified Hh proteins are expected to be tightly anchored to the cell membrane. Therefore, a key issue in Hh signalling is to understand how the lipid-modified Hh is released from its site of synthesis and subsequently moves through a field of cells.

Hh signalling is essential for many developmental processes in Drosophila (Ingham and McMahon, 2001). The role of Hh is particularly well documented during wing disc development. In the wing disc, Hh is expressed in the posterior (P) compartment and moves anteriorly to transduce its signal in a narrow stripe of tissue in the anterior compartment (A) abutting the anteroposterior (AP) boundary. Hh signalling induces the expression of its target genes including decapentaplegic (dpp), patched (ptc), collier (col) and engrailed (en) in a concentration-dependant manner (Chen and Struhl, 1996; Mullor et al., 1997; Strigini and Cohen, 1997; Vervoort et al., 1999, 1999; Zecca et al., 1995). Although Dpp diffuses bidirectionally into both compartments and functions as a long-range morphogen to control the growth and patterning of cells in the entire wing (Lecuit et al., 1996; Nellen et al., 1996), Hh signalling is required for the proper patterning of the region near the AP compartment boundary (Chen and Struhl, 1996; Strigini and Cohen, 1997; Vervoort et al., 1999). Genetic screens in Drosophila have identified three components including Patched (Ptc), Dispatched (Disp) and Tout-velu (Ttv), which play distinct roles in Hh distribution. Disp and Ptc are multi-span transmembrane proteins and essential for Hh trafficking in its producing cells and receiving cells, respectively. Disp is required in Hh-producing cells and it appears to control the release of Hh-Np from the producing cells (Burke et al., 1999). In the absence of Disp function, Hh-Np accumulates in its producing cells and fails to move into anterior receiving cells (Burke et al., 1999). The distribution of Hh in its receiving cells is regulated by its receptor Ptc that is expressed in all A compartment cells and is upregulated by Hh signalling (Forbes et al., 1993; Goodrich et al., 1996; Marigo et al., 1996). Upregulated Ptc protein in the Hh-responding wing cells sequesters Hh and thereby restricts the further movement of Hh into the A compartment (Chen and Struhl, 1996).
Genetic studies of the function of Ttv in Drosophila have demonstrated an essential role of heparan sulphate proteoglycan(s)(HSPG) in Hh movement in its receiving cells (Bellaiche et al., 1998; The et al., 1999). Ttv is a Drosophila homolog of the mammalian EXT1 tumor suppressor gene (McCormick et al., 1998) encoding a heparan sulphate co-polymerase (Lind et al., 1998; The et al., 1999). HSPGs are cell surface macromolecules consisting of a protein core and a number of heparan sulphate glycosaminoglycan (HS GAG) chains (Bernfield et al., 1999; Lin and Perrimon, 2000; Perrimon and Bernfield, 2000). In Drosophila, there are two glypicans, division abnormally delayed (daily) (Nakato et al., 1995) and dally-like (dly) (Baeg et al., 2001; Khare and Baumgartner, 2000), one Drosophila syndecan (dsyndecan) (Spring et al., 1994), and one perlecanc encoded by terribly reduced optic lobes (trol) (Park et al., 2003; Voigt et al., 2002). Glypicans represent one of main cell surface HSPGs, which linked to the plasma membrane by a glycosyl phosphatidylinositol (GPI) linker (Bernfield et al., 1999; Lin and Perrimon, 2000; Perrimon and Bernfield, 2000). Previous studies have demonstrated that the movement of Hh from P to A compartment is defective in cells mutant for tout-velu (ttv) (Bellaiche et al., 1998). Cholesterol-unmodified Hh-N, but not Hh-Np, is independent of Ttv function for its movement (The et al., 1999). A recent study further showed that cholesterol in Hh-Np is required for the formation of large punctate structures in embryonic epidermis, whereas the movement of these large punctate structures across cells is contingent upon the activity of Ttv (Gallet et al., 2003). Together, these studies suggest that the proteoglycan(s) modified by Ttv is specifically required for the movement of cholesterol-modified Hh-Np. However, the precise function of HSPG(s) in regulating Hh movement and its signalling is not understood. In particular, it is currently unknown which class of HSPGs is involved in Hh signalling.

In this report, we provide evidence that Dally and Dly, two glypicin members of HSPGs, are the substrates for Ttv and are involved in Hh movement and its subsequent signalling. We show that Dly is required for Hh signalling during embryogenesis, and that both Dally and Dly are required and redundant in Hh movement during wing disc development. Importantly, we found that a narrow strip of cells defective in HS GAG biosynthesis or mutant for daily-dly severely impaired the further movement of Hh to more anterior cells in the wing disc, suggesting that HSPG-mediated Hh movement through a field of cells is regulated by a cell-to-cell mechanism rather than by free diffusion. We further demonstrate that Hh movement is independent of dynamin-mediated endocytosis. These findings led us to propose that the glypicans Dally and Dly transfer Hh along the cell membrane to pattern a field of cells.

Materials and methods

Drosophila stocks

sffB4 is a null allele for sff as described (Baeg et al., 2001; Lin and Perrimon, 1999). hhB1, PGr41, hhJ13 and shpJ1 are described in FlyBase. UAS-GFP-Dpp and dppGal4 stocks are described elsewhere (Entchev et al., 2000; Telemann and Cohen, 2000). dpp-LacZ is a lacZ-expressing enhancer trap allele of dpp (dpp-lacZ2/10638) (Jiang and Struhl, 1995). m6 contains a nonsense mutation at AA224 (224 R to stop) isolated from a screen described (Belenkaya et al., 2002). UAS-GFP-Dly contains the Wg signal peptide (AA1-AA37) followed by GFP protein fused with Dly in which the signal peptide (AA1 to AA 42) of Dly is deleted. The UAS-GFP-Dly can rescue the cuticle defect of dlyA187 null embryo (data not shown), thus is functional.

Generation of daily and dly null alleles

daly80 was generated by P-element mediated mutagenesis using dailyP1 (Nakato et al., 1995). daily80 contains a deletion from -724 to +415 (A in the ATG start codon is designated as 1) that covers the ATG start codon followed by 54 amino acids including the signal peptide as well as part of the first intron. dlyA187 is an EMS-induced mutant that was isolated from a deficiency screen using Df(3L)fz-M21. dlyA187 contains a deletion of 26 nucleotides resulting in a reading frame shift from amino acid 205. dlyA187 lacks part of the cysteine-rich region, the entire GAG attachment domain and the glycosylphosphatidylinositol (GPI)-anchoring signal (Lin and Perrimon, 2000; Perrimon and Bernfield, 2000).

Wing phenotype analysis

Wing phenotypes were analyzed by directed mosaic (Duffy et al., 1998) using UAS-Flipase(flp)/vgQ1206-Gal4 as described (Belenkaya et al., 2002). Males of genotype w; daly80 (or dlyA187 or daily80) dlyA187 FRT2A/TM6B were crossed with females of genotype w; vg Q1206-Gal4 UAS-fplp; FRT2A/TM6B that express fplp primarily in the wing imaginal disc cells under vg Q1206-Gal4 control. This fplp activity mediates a high frequency of mitotic recombination, generating clones of cells homoygous for daily80 or dlyA187 or daily80,dlyA187.

Generation of germline clones and marked wing clones

daly germline clones were generated by the autosomal FLP-DHS technique (Chou and Perrimon, 1996). Clones of mutant cells were generated by the FLP-FRT method (Golic, 1991; Xu and Rubin, 1993) and induced by subjecting first- or second-instar larvae to a heat shock at 37°C for 2 hours. To generate shibire mutant clones, larvae were allowed to grow at 18°C and were shifted to 32°C for 5 or 10 hours prior to fixation and antibody staining. The genotypes used in our analyses were as follows.

1. dlyA187 germline clones (Fig. 1): y w hspt0-flp; dlyA187 FRT2A/P[ovOD]FRT2A × dlyA187 FRT2A/TM6B.
2. daily80,dlyA187 clones marked by the absence of GFP (Fig. 2): y w hspt0-flp/+ or Y; hspt0-Myc-GFP M(3)i 55 FRT2A/daily80,dlyA187 FRT2A.
3. sfB4 clones marked by the absence of GFP using dpp-LacZ (Fig. 4): y w hspt0-flp/+ or Y; dpp-LacZ/+; ubiquitin-GFP FRT2A/sfB4 FRT2A.
4. Wing discs expressing GFP-Dpp by dppGal4 and having sfB4 or daily80,dlyA187 clones marked by the absence of pMyo (Fig. 4): y w hspt0-flp/+ or Y; UAS-GFP-Dpp/+; hs-Myo FRT2A/sfB4 (or daily80,dlyA187) dppGal4 FRT2A.
5. The wing disc expressing GFP-Dpp by dppGal4 and having itv63 mutant cells marked by the absence of lacZ (Fig. 4): y w hspt0-flp/+ or Y; UAS-GFP-Dpp FRTG13 armadillo-LacZ/FRTG13 tv63; dppGal4/+.
6. shiB4 clones marked by absence of LacZ (Fig. 5): armadillo-LacZ FRT18A/shiB4 FRT18A hspt0-flp/+.
7. A sfB4 clone marked by the absence of GFP (Fig. 6): y w hspt0-flp/+ or Y; ubiquitin-GFP FRT2A/sfB4 FRT2A.

Immunostaining, in situ hybridization and western blot analysis

All imaginal discs shown in this report were dissected from mid-late third instar larvae, fixed and stained with appropriate primary antibodies to mark clones and monitor target-gene expression or Hh distribution after the induction of clonal markers as described (Belenkaya et al., 2002; Lin and Perrimon, 1999). Primary antibodies were mouse anti-En (1:1000) (Patel et al., 1989), rabbit anti-Hh (1:2000) (Taylor et al., 1993), rat anti-Ci (1:10) (Motzny and
Holmgren, 1995), mouse anti-Ptc (Capdevila and Guerrero, 1994), rabbit anti-β-Col (Vervoort et al., 1999), rabbit anti-β-Gal (1:500) (Cappel), mouse anti-β-Gal (1:3000), mouse anti-Myc (1:500) (Roche), rabbit anti-GFP (Clonetech) and mouse anti-GFP (Chemicon). The primary antibodies were detected by fluorescent-conjugated secondary antibodies from Jackson ImmunoResearch Laboratories. Standard protocol was used for in situ hybridization. (H.I) bap transcription in stage 10 wild-type (H) and dlyA187 mutant (I) embryos visualized by in situ hybridization.

Results

Dly is required for Hh signalling during embryogenesis

To dissect the molecular mechanism(s) by which HSPGs(s) regulates Hh signalling, we first sought to identify specific proteoglycan(s) involved in Hh signalling during embryonic patterning. During embryogenesis, Hh and Wingless (Wg) are expressed in adjacent cells and are required for patterning of epidermis (Siegfried and Perrimon, 1994). In stage 10 embryos, Hh is expressed in two rows of cells in the posterior compartment of each parasegment, while Wg is expressed in one row of cells anterior to Hh expression cells. The expression of Hh is controlled by Engrailed (En) whose expression is maintained by Wg signalling through a paracrime regulatory loop. Hh signalling in turn is required for maintaining the expression of Wg whose activity controls the production of the naked cuticles (Hatini and DiNardo, 2001). Loss of either Hh or Wg signalling leads to a loss of naked cuticle, which is defined as segment polarity phenotype.

Our previous study showed that disruption of dly in embryos by RNA interference (RNAi) led to a strong segment polarity defect, suggesting that Dly is likely to be involved in Hh and/or Wingless (Wg) signalling in embryonic epidermis (Baeg et al., 2001). To explore the potential role of Dly in Hh signalling, we isolated a number of dly mutant alleles using EMS mutagenesis. dlyA187 is a null allele (see Materials and methods for details) and is used for further analyses. Animals zygotically mutant for dly appears to have normal cuticle patterning (data not shown) and survive until third instar larvae. However, homozygous mutant embryos derived from females lacking maternal dly activity (referred to as dly embryos hereafter) die with a strong segment-polarity phenotype (Fig. 1C) resembling those of mutants of the segment polarity genes hh (Fig. 1B) and wg (data not shown). In dly embryos, both En expression and wg transcription fade by stage 10 (Fig. 1E,G), suggesting further that dly is involved in the Hh and/or Wg pathways.

To further determine whether Dly activity is required for Hh signalling in embryogenesis, we examined Hh signalling activity in dly embryos during mesoderm development. Hh and Wg signalling have distinct roles in patterning embryonic mesoderm. Hh signalling activates the expression of a mesodermal specific gene bap (in the anterior region of each parasegment, whereas Wg signalling inhibits bap expression in the posterior region (Azpiazu et al., 1996). bap expression is diminished in the hh mutant, but is expanded to the posterior parasegment in the wg mutant (Azpiazu et al., 1996). Consistent with a role of Dly in Hh signalling, we found that bap expression was strikingly reduced in dly embryos (Fig. 1I). Together with the segment polarity phenotype, these results strongly argue that Dly is required for Hh signalling during embryogenesis.

dally and dly are required for Hh signalling in wing development

We further examined the role of Dly in Hh signalling during wing development in which Hh and Wg signalling function independently of each other. In the wing disc, Hh signalling induces the expression of its target genes in a narrow stripe of tissue in the A compartment abutting the AP boundary. Hh signalling patterns the central domain of wing blade and controls the positioning of longitudinal veins L3 and L4 (Mullor et al., 1997; Slusarski et al., 1995; Strigini and Cohen, 1997). We first examined the roles of Dly in Hh signalling by analyzing adult wing defects using ‘directed mosaic’ technique (Belenkaya et al., 2002; Duffy et al., 1998) (see Materials and methods for details). To our surprise, we did not observe any detectable phenotypes in adult wings bearing dly mutant clones (data not shown). We reasoned that Hh signalling may be mediated by other HSPGs in the wing. One candidate is the glypcian dally (Nakato et al., 1995) that has previously been
shown to be involved in Wg (Lin and Perrimon, 1999; Tsuda et al., 1999) and Dpp signalling (Jackson et al., 1997). Because available 
dally alleles used previously were hypomorphic, we
generated several 
dally null alleles by P-element mediated
mutagenesis. 
dally is a null allele and was used for our
analysis (see Materials and methods for details). However,
similar to other 
dally alleles, homozygous 
dally animals are
viable. The wing bearing 
dally clones exhibits a partial loss of the
L5 vein with a high penetrance, but no detectable defects
in the central domain of wing blade (arrow in Fig. 2B). To
determine whether 
dally and dly have overlapping roles in Hh
signalling in wing development, we generated clones mutant
for both 
dally and dly (referred as 
dally-dly hereafter). Interestingly, the adult wings bearing clones mutant for 
dally-dly show L3-L4 fusion (arrow in Fig. 2C). This phenotype
typical of loss of Hh function, suggesting that 
Dally and Dly
play redundant roles in Hh signalling in wing development.

We further examined the effect of 
dally-dly on Hh signalling
by directly analyzing the expression of Hh target genes
including
ptc, en and col. The following experiments provide
compelling evidence for the involvement of Dally and Dly
in Hh signalling in the wing disc. First, in the wild-type wing disc,
Ptc expression is normally elevated by Hh signalling in anterior
cells adjacent to AP boundary (Fig. 2D). Levels of Ptc
expression are reduced in 
dally-dly clones (Fig. 2E, E’, F’). Second, En expression is induced by Hh signalling
over three to four cell diameters in anterior cells adjacent to the
AP boundary (Fig. 2G) (Blair, 1992). This induction is eliminated
in 
dally-dly clones (Fig. 2H, H’). Third, Col is normally
expressed in four cell diameters adjacent to the AP boundary
(Fig. 2I) (Vervoort et al., 1999). In the 
dally-dly clone, Col
expression is reduced and limited to two rows of cells at the
posterior edge of the clone and with a lower level in the more
anterior row (Fig. 2J, J’). Collectively, these observations argue
that Dally and Dly
are required and are redundant in Hh
signalling in the wing disc.

**Ttv affects HS GAG modifications in Dally and Dly**

Previous studies have demonstrated the involvement of Ttv
in Hh signalling (Bellaiche et al., 1998; The et al., 1999). 
ttv is a 
Drosophila
homolog of the mammalian 
EXT1 gene encoding
the HS co-polymerase required for the biosynthesis of HS
GAG chains (Lind et al., 1998; The et al., 1999). We reasoned
that if Dally and Dly
are involved in Hh signalling, they may
be modified by Ttv. We therefore examined the modification
of Dally and Dly
in 
ttv mutant larvae by western blot analysis.

---

**Fig. 2.** 
dally and dly are required for Hh signalling in wing development.

(A-C) Wings are oriented proximal towards the left and anterior upwards.

(A) Wild-type wing with labeled
longitudinal veins. (B) A wing carrying
clones of 
dally showing a partial L5 vein loss (arrow) (80%, n = 100). (C) A wing carrying clones of 
dally mutant cells showing L3-L4 vein fusion
(arrow) (10%, n = 80). (D-J) All the wing
discs were derived from third instar larvae. Anterior is towards left and
dorsal is upwards in all the wing disc images. (D-F) Ptc expression (red) in a
wild-type wing disc (D) and a disc
carrying a 
dally clone (E, E’, F’). In wild-type disc, Ptc is
expressed in a stripe of cells abutting the
AP boundary in response to Hh from the
P compartment. Ptc expression is
normally elevated by Hh signalling in anterior
larvae. Anterior is towards left and
dorsal is upwards. (A-C) Wings are oriented proximal
(towards the left and anterior upwards.)

In wild-type disc, Ptc is
expressed in a stripe of cells abutting the
AP boundary in response to Hh from the
P compartment. Ptc expression is
normally elevated by Hh signalling in anterior
larvae. Anterior is towards left and
dorsal is upwards.
As shown previously (Baeg et al., 2001; Giraldez et al., 2002; Lin and Perrimon, 1999; Tsuda et al., 1999), in wild-type larval extracts, both Dally and Dly migrate as bands with high molecular masses, characteristic of HSPGs to which negatively charged HS GAG chains are attached (Fig. 3A,B). In homozygous tv larvae, the high molecular mass of Dly is shifted to a relatively lower molecular mass. The high molecular mass of Dally is also significantly reduced. These results indicate that both Dally and Dly are probably the substrates of Ttv.

**Hh moves from cell to cell**

Previous studies on Ttv suggest that HSPG(s) is required for Hh movement in receiving cells (Bellaiche et al., 1998; The et al., 1999). To further define the molecular mechanism(s) of this requirement, we examined Hh signalling defects in clones mutant for sulfateless (sfl), which encodes a heparan sulphate N-deacetylase/N-sulfotransferase required for HS GAG biosynthesis (Lin et al., 1999; Lin and Perrimon, 1999). Mutations in sfl are expected to impair all of the functions of HSPGs. Consistent with previous studies in ttv (Bellaiche et al., 1998), we observed a strong reduction in dpp-lacZ expression in sfl mutant clones located in anterior cells adjacent to AP boundary. However, dpp-lacZ expression can be detected in one to two posterior-most rows of cells in the clones (Fig. 4B,C). Surprisingly, the dpp-lacZ expression is absent in anterior wild-type cells even at the places where the adjacent clone is only one cell-diameter width (arrows in Fig. 4B,C). As β-galactosidase (β-gal) is a relatively stable protein, to eliminate the possibility of perdurance of β-gal, we used UAS-GFP-dpp (Entchev et al., 2000) under the control of dpp Gal4 to confirm the above findings. Indeed, GFP-Dpp expression is restricted to the posterior-most row of cells in the mutant clones. Hh movement towards anterior cells is blocked by even one cell diameter of mutant clones (arrows in B,C,E,H) as indicated by dpp-lacZ or GFP-Dpp expression.
Fig. 5. Hh movement is independent of dynamin-mediated endocytosis. All the wing discs were derived from third instar larvae. Anterior is towards left and dorsal is upwards in all the wing disc images (A–E′). In all discs, the shi ts1 clones are marked by the absence of β-gal staining (green) and outlined with broken lines. The AP boundaries are determined by Ci staining (not shown) and are marked by solid lines. (A–C) Hh staining in a wild-type wing disc (A) and a disc carrying a shi ts1 clone in the A compartment (B,C). Hh protein appears to be graded membrane staining and is concentrated in large punctate particles (indicated by arrows) in three or four cell diameters abutting AP boundary in wild-type disc (A). These large punctate particles are absent inside the shi ts1 clone and can only be observed at the boundary between mutant and wild-type cells (arrows in B). (B′) The merged image stained with Hh and lucZ. (C) A basal optical section of the same disc shown in B where Hh accumulation along the cell membrane in the clone is more prominent. (D,D′) Ptc staining in a disc carrying shi ts1 clones at AP boundary. Ptc expression is expanded anteriorly within the lower larger clone. Noticeably, the wide-type cells anterior to the upper small clone still express Ptc, suggesting that shi ts1 cells cannot block Hh movement. (E,E′) Ci staining in a disc carrying shi ts1 clones. Ci expression pattern is not significantly altered in the clones.

Hh movement is independent of dynamin-mediated endocytosis

The cell-to-cell movement of Hh could be mediated by endocytic routes through planar transcytosis (Bejsovec and Wieschaus, 1995; Moline et al., 1999; Seto et al., 2002) or by movement along the cell surface. To determine whether Hh movement is mediated by planar transcytosis, we examined Hh distribution and its subsequent signalling in clones mutant for movement. We further limited to the posterior-most row of A cells within sfl mutant clones (Fig. 4E,F). We also found that only one cell-diameter width of the sfl mutant clone within the Dpp expression domain is sufficient to completely block Dpp-GFP expression in the adjacent anterior wild-type cells (an arrow in Fig. 4E). This phenotype is fully penetrant and independent of the position of sfl mutant cells. We interpret this observation as an inability of sfl mutant cells to relay Hh molecules to more anterior cells even though they are capable of receiving and transducing Hh signal. We also observed the same single-row-blockage phenomenon in ttv and dally-dly clones (Fig. 4G,H) albeit at lower frequency in dally-dly (20%, n=49). Taken together, these results suggest that the HSPG-dependent Hh movement is through a cell-to-cell mechanism.

We further examined the activity of Hh signalling in clones mutant for shi ts1. Both Ptc and Ci expression levels are not reduced in clones mutant for shi ts1 (Fig. 5D,D′,E,E′), even when the incubation at 32°C was extended to 10 hours (data not shown). Instead, we found that Ptc expression is expanded within the large shi ts1 clone (Fig. 5D,D′), which are presumably internalized vesicles containing both Hh and Ptc, and are readily seen in three to four rows of A cells abutting the AP boundary in wild-type disc (Fig. 5A, arrows).

HSPGs co-localize with and stabilize Hh

One possible role of HSPGs in Hh movement is to stabilize Hh on the cell surface by directly interacting and forming complexes with Hh. We tested this by examining the co-localization of Hh with Dly. We ectopically expressed a GFP-Dly fusion protein in either the ptc domain or the hh domain in wing discs. Hh co-
localizes with GFP-Dly in both cases as shown by diffusive membrane staining and punctate particles (Fig. 6A,B). This finding is also consistent with our following loss-of-function analyses. We examined Hh distribution in sfl mutant cells in the wing disc as well as in dly embryos. Hh staining disappears in sfl mutant clones, except at a residual level in the posterior-most row of cells (Fig. 6C). In wild-type embryos, Hh staining is detected as punctate particles, which are found at least one cell diameter from its producing cells (En-positive cells) (Fig. 6D) (Porter et al., 1996a; Tabata and Kornberg, 1994; Taylor et al., 1993). However, in dly embryos, these punctate particles disappear and Hh staining appears to be membrane bound in its producing cells (Fig. 6E). Similar defects in Hh distribution were also observed in tv embryos (Gallet et al., 2003; The et al., 1999). Together, these observations suggest that glypicans co-localize with Hh and regulate Hh movement possibly by forming complex(es) with Hh and stabilizing Hh protein on the cell surface.

Discussion

Hh signalling controls many developmental processes in animal development. Here, we demonstrate essential functions of the Drosophila glypicans Dally and Dly in Hh signalling and its movement. Our results extend previous findings that Hh movement is controlled by Ttv, the HS co-polymerase required for the biosynthesis of HSPGs. Importantly, we further demonstrate that glypican-mediated Hh movement is regulated by a cell-to-cell mechanism that is independent of dynamin-mediated endocytosis. Together, our results suggest that the glypicans Dally and Dly transfer Hh along the cell membrane to pattern a field of cells.

Role of Dly in Hh signalling during embryogenesis

This study first demonstrates that Dly is the main HSPG involved in Hh signalling during embryogenesis, at least in epidermis and mesoderm, the two tissues that were carefully examined. Three lines of evidence strongly support our conclusion. First, embryos lacking both maternal and zygotic dly activities develop a strong segment polarity defect and exhibit diminished expression of En and Wg (Fig. 1C,E,G). Second, we found that Hh can be detected as punctate particles at least one cell diameter from its producing cells and these punctate particles are absent in dly-null embryos (Fig. 6D,E). Third, we observed a reduced expression of bap in dly mutant embryos (Fig. 1I), a phenotype specifically attributed to the Hh signalling rather than Wg signalling defect. Previously, it was shown that the punctate particles of Hh staining are absent in tv null embryos (The et al., 1999). Recently, Gallet et al. showed that the formation of such Hh staining particles, referred as large punctate structures (LPS), requires cholesterol modification (Gallet et al., 2003). They further demonstrated that movement of these large punctate structures across cells is dependent on Ttv activity (Gallet et al., 2003). Our results are consistent with these observations and suggest that Dly is the main HSPG involved in the movement of these LPS across cells. It is conceivable that the punctate particles of Hh staining we observed may represent Hh-Dly complexes. In this regard, Dly may either prevent secreted Hh from being degraded and/or facilitate Hh movement from its expression cells to adjacent receiving cells. These two mechanisms are not mutually exclusive. In the absence of Dly function, secreted Hh is either degraded or fails to move to the adjacent cells.

In addition to dly, three other HSPGs, including Dally, Dsyndecan and Trol, are also expressed in various tissues during embryogenesis. In particular, dally is expressed in epidermis and has been shown to be involved in Wg signalling (Lin and Perrimon, 1999; Toyoda et al., 2000). Removal of Dally activity in embryos either by dally hypomorphic mutants or by RNA interference (RNAi) generates denticle fusions (Lin and Perrimon, 1999; Toyoda et al., 2000). Further studies demonstrated that the cuticle defect associated with dally embryos by RNAi is weaker than that of dly (Baeg et al., 2001).

Our results in this work suggest that Dly plays more profound roles in embryonic patterning than Dally. It remains to be determined whether Dally and other two Drosophila HSPGs are involved in Hh signalling in other developmental processes during embryogenesis.
Role of Dally and Dly in Hh movement during wing development

We further demonstrate that Dally and Dly are involved and are redundant in Hh signalling in the wing disc. Consistent with this, we also show that the GAG chains of Dally and Dly are altered in the absence of Ttv activity, suggesting that both Dally and Dly are indeed the substrates for Ttv (Fig. 3A,B). Redundant roles of cell membrane proteins have been demonstrated in many other signalling systems. For example, both Frizzled (Fz) and Drosophila Frizzled 2 (Fz2) are redundant receptors for Wg (Bhanot et al., 1999; Chen and Struhl, 1999), although Fz2 has relative high affinity in binding to Wg protein (Rulifson et al., 2000). Dly protein is distributed throughout the entire wing disc (data not shown). Previous studies demonstrated that dally is highly expressed at the AP border (Fujise et al., 2001). Interestingly, Dally expression at the AP border is overlapped with the ptc expression domain and is under the control of Hh signalling (Fujise et al., 2001).

It is likely that both Dally and Dly are capable of binding to Hh and facilitating the movement of the Hh protein. In the absence of one of them, another member is probably sufficient to facilitate Hh movement.

We notice that dally-dly double mutant clones have relatively weaker defects in Hh signalling in the wing disc than those of the ttv and sfl mutants. One possible explanation is the perdurance of Dally and Dly proteins. Alternatively, two other HSPGs, Dsyndecan and Trol, may also participate in Hh signalling in the absence of Dally and Dly in the wing disc. These issues remain to be examined using both dsyndecan and trol null mutants.

Role of HSPGs in Hh signal transduction

Do HSPGs act as co-receptors in Hh signal transduction? Hh is a heparin-binding protein and is likely to interact with HSPGs through their HS GAG chains. In support of this, we show that Dly colocalizes with Hh punctate particles (Fig. 6A,B). It is conceivable that Dally and Dly could either transfer Hh to its receptor Ptc or form a Hh-Dally/Dly-Ptc ternary complex in which Dally and Dly may function to facilitate Hh-Ptc interaction or stabilize a Hh-Ptc complex. In this regard, Dally and Dly may function both in transporting Hh protein and acting as co-receptors in Hh signalling (Lin and Perrimon, 2003). Consistent with this view, a recent report using RNAi in tissue culture based assays identified Dly as a new component of the Hh pathway (Lum et al., 2003). It was shown that Dly plays a cell-autonomous role upstream or at the level of Ptc in activating the expression of Hh responsive-reporter, suggesting a role of Dly in the delivery of Hh to Ptc.

It is important to note that some of results obtained from tissue culture based assays (Lum et al., 2003) are not consistent with our in vivo results reported here as well as previous studies on Ttv (Bellaiche et al., 1998; The et al., 1999). Cl-8 cells were originally derived from the wing disc. However, we found that removal of dly activity alone has no detectable effect on Hh signalling in the wing disc. This apparent discrepancy may due to several factors. First, Hh-N, instead of Hh-Np was used as a source for Hh in their work. Second, Cl-8 cell may have altered the proteoglycan expression pattern, which can be significantly different from Hh-responding wing cells in which Dally expression is upregulated by Hh signalling (Fujise et al., 2001). Finally, it is possible that Dly may have a higher capacity than Dally to bind Hh, as in the case for Wg (Baeg et al., 2001). In this regard, removal of Dly will probably lead to more profound effects than removal of other HSPGs on binding of Hh-N to the cell surface, perhaps in the delivery of Hh-N to Ptc.

We notice that within sfl, or ttv or dally-dly mutant clones, the posterior-most cells adjacent to wild-type cells are still capable of transducing Hh signalling (Fig. 4). It is most likely that Hh proteins bound by Dally and Dly in wild-type cells can directly interact with Ptc located on the cell surface of the adjacent mutant cells to transduce its signalling. In support of this view, Strigini and Cohen previously demonstrated that an Hh-C/2 membrane fusion protein can activate Hh signalling in its adjacent cells (Strigini and Cohen, 1997). Furthermore, studies on Disp have shown that the first row of anterior cells adjacent to posterior Hh-producing cells have significant Hh signalling activity in disp mutant wing disc, in which Hh is retained on the cell surface of its producing cells (Burke et al., 1999; Ma et al., 2002). Interestingly, we also observed Hh punctate particles in the posteriormost HSPG mutant cells adjacent to wild-type cells (Fig. 6C). These Hh punctate particles are most likely intracellular Hh proteins internalized through Ptc mediated endocytosis process. In this regard, HSPGs may not be required for Ptc-mediated Hh internalization.

Mechanism(s) of Glypican-mediated Hh movement

Recent biochemical studies from vertebrate cells have shown that Shh-Np is secreted from cells and can be readily detected in conditioned culture medium (Zeng et al., 2001). It was also shown that overexpression of Disp can increase the yield of Hh protein in the culture medium (Ma et al., 2002). These experiments suggest that Hh can be directly secreted from its expression cells. Can secreted Hh proteins freely diffuse to its receiving cells through extracellular spaces? To address this issue, we have conducted detailed analyses for Hh signalling in the complete absence of HS GAG using sfl and ttv or absence of glypicans using dally-dly. We show that a narrow strip (one cell diameter in width) of sfl or ttv, or dally-dly mutant cells prevents the transpassing of the Hh signal (Fig. 4). We also show that Hh staining disappears in sfl mutant clones, except at a residual level in the posterior-most row of cells (Fig. 6C). Based on these observations, we favour a model in which Hh movement is regulated by a cell-to-cell mechanism rather than by free diffusion.

Our results further suggest that Hh movement is independent of dynamin-mediated endocytosis which has been shown to be involved in the transportation of morphogen molecules (Seto et al., 2002) such as Dpp (Entchev et al., 2000) and Wg (Bejsovec and Wieschaus, 1995; Moline et al., 1999). We found that a blockage of dynamin function did not eliminate Hh movement and its subsequent signalling; instead, it led to a striking reduction of punctate particles of Hh staining and an accumulation of cell-surface Hh protein (Fig. 5). We also observed expanded Ptc expression domain when dynamin-mediated endocytosis is blocked (Fig. 5). These new findings provide compelling evidence that dynamin-mediated endocytosis is not required for Hh movement and its subsequent signalling, but is involved in Ptc-mediated internalization of the Hh protein.
Fig. 7. Model of Hh movement. The orange arrow indicates that Disp releases Hh proteins from the cell membrane of the Hh-producing cells. The purple arrow indicates that glypicans can present Hh to Ptc. The thin black arrows at the top of this diagram indicate the displacement of Hh from one GAG chain to another GAG chain. The double-headed arrow indicates the lateral movement of glypicans on cell membrane.

A model of Hh movement
Several mechanisms have been proposed to explain morphogen transport across a field of cells. These mechanisms include (1) free diffusion, (2) active transport by planar transcytosis, (3) cytonemes, (4) argosomes (Entchev et al., 2000; Ramirez-Weber and Kornberg, 1999; Telemán et al., 2001; Vincent and Dubois, 2002). Our results suggest that Hh moves through a cell-to-cell mechanism rather than free diffusion. Furthermore, we demonstrate that dynamin-mediated endocytosis is unlikely to be involved in Hh movement. On the basis of our findings, we propose the following model by which the HSPGs Dally and Dly may regulate the cell-to-cell movement of the Hh protein across a field of cells. In this model (Fig. 7), Hh is released by Disp from its producing cells and is immediately captured by the GAG chains of glypicans on the cell surface. The differential concentration of Hh proteins on the surface of producing cells and receiving cells drives the unidirectional displacement of Hh from one GAG chain to another towards more distant receiving cells. Within the same cell, the transport of Hh may be facilitated by the lateral movement of glypicans on the cell membrane. On the receiving cells, glypicans may present Hh to Ptc, which then mediates the internalization of Hh. Glypican mutant cells can not relay Hh proteins further as they lack HS GAG on the surface. However, they are able to respond to the Hh signal because Ptc may contact the Hh on the membrane of the adjacent wild-type cells. Further studies are needed to determine whether other mechanism(s) including cytonemes and argosomes are also involved in Hh movement.

We thank S. Cohen, S. Eaton, M. González-Gaitán, I. Guerrero, R. Holmgren, P. Ingham, J. Jiang, P. O’Farrell, G. Struhl, A. Vincent, the Developmental Studies Hybridoma Bank and Bloomington stock center for reagents; R. Converse, D. Houston, R. Lang, H. Standley and A. York for comments on the manuscript; and H. Liu for technical assistance. This work is supported by a NIH grant GM63891, a grant from Ohio Cancer Associates and a start-up fund from Cincinnati Children Hospital Research Foundation to X.L. C.H. is an Albert J. Ryan fellow and a American Heart Association predoctoral fellow.

References
Chen, M. S., Obar, R. A., Schroeder, C. C., Austin, T. W., Poodry, C. A.,


Glypican in Hedgehog movement and signaling


