The glypican Dally-like is required for Hedgehog signalling in the embryonic epidermis of Drosophila

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Introduction

Animal development is under the control of a handful of signalling pathways that are activated by extracellular ligands, the most prominent activating the Hedgehog (Hh), Wnt, FGF, EGF, TGFβ/BMPs or Notch pathways. The secretion, movement and reception of these extracellular signals is tightly regulated, and recent work has implicated the heparan sulphate proteoglycans (HSPGs) in the regulation of ligand activity (Bernfield et al., 1999; Perrimon and Bernfield, 2000; Selleck, 2000; Turnbull et al., 2001). Two types of evidence indicate that HSPGs play a role in signalling: first, extracellular ligands are often found tightly associated with the cell surface, and this association can be inhibited by heparin, a subclass of heparan sulphate. Second, mutations in enzymes involved in the biosynthesis of heparan sulphate chains impair specific signalling events in vertebrate and invertebrate development. In Drosophila, the gene sugarless (sfl) codes for a UDP glucose dehydrogenase required for the synthesis of heparan sulphate chains, and mutations in sfl disrupt both Wingless (Wg, homologue of Wnt-1) and FGF signalling in the embryo (Binari et al., 1997; Hacker et al., 1997; Haerry et al., 1997; Lin et al., 1999; Toyoda et al., 2000). Mutations in sulfateless (sfl), which codes for a N-deacetylase/N-sulphotransferase required for the sulphation of heparan sulphate chains, also disrupts Wg and FGF signalling in embryos (Lin et al., 1999; Lin and Perrimon, 1999; Toyoda et al., 2000). tout-velu (tvu) codes for a co-polymerase required for the elongation of heparan sulphate chains (Bellaiche et al., 1998; The et al., 1999; Toyoda et al., 2000). tvu mutations disrupt the movement of Hh in wing discs, and are thought to disrupt Hh signalling but not Wg or FGF signalling in embryos (Bellaiche et al., 1998; The et al., 1999). More recently, mutations in fringe, a gene coding for a UDP sugar transporter, were shown to disrupt Wg or Hh signalling in embryos, as well as FGF signalling (Selva et al., 2001; Goto et al., 2001). Given their pleiotropic effects, however, it is difficult to use these mutants to determine at which step of a given signalling pathway HSPGs intervene.

One avenue of research is to identify the protein cores of HSPGs that are required for each signalling pathway. Two types of proteoglycans bear the majority of heparan sulphate chains at the cell surface: the syndecans and the glypicans (Bernfield et al., 1999). Mutations in the latter produce developmental defects in mice, zebrafish and Drosophila, making glypicans good candidates for having a role in signalling (Bernfield et al., 1999; Perrimon and Bernfield, 2000; Selleck, 2000; Song and Filmus, 2002). Glypicans are glycosylphosphatidylinositol (GPI)-anchored proteins, which are thought to be permanently glycanated, and that carry several heparan sulphate chains linked to serine residues adjacent to the plasma membrane. The Drosophila genome contains two glypicans: daily and daily-like (dlp). Daily has been implicated in the regulation of Wg and Dpp signalling, and Dlp in the regulation of Wg signalling (Jackson et al., 1997; Lin and Perrimon, 1999; Tsuda et al., 1999; Baeg et al., 2001).

We have tested the requirement of the two Drosophila glypicans for Wg and Hh signalling in the embryonic epidermis. Wg is a secreted glycoprotein that activates the receptors Frizzled and Frizzled2, which then turn on a downstream signalling cascade leading to the activation or repression of target genes (Wodarz and Nusse, 1998). The full-length Hh protein undergoes an autocatalytic processing in the secreting cells and is further modified by addition of two lipids,
a cholesterol and a palmitoyl moiety. Hh binds its receptor Patched (PtC) on the receiving cells, and this relieves PtC inhibition on another transmembrane protein, Smoothened (Smo), which in turn transduces the Hh signal (Ingham and McMahon, 2001). Wg and Hh are expressed in stripes in the embryonic segments and their functions in epidermal patterning are now well understood (Martinez Arias, 1993; Hatini and Dinardo, 2001; Sanson, 2001). Early in embryogenesis, Wg is required to maintain the transcription of engrailed (en) in adjoining cells. Once the expression of en becomes independent of Wg, around stage 11, Wg is required for the specification of cells that secrete a smooth or ‘naked’ cuticle, through the repression of the gene shavenbaby. The en-expressing cells secrete Hh, which in turn is required to maintain the transcription of wg, its main target in the epidermis. Thus wg, en and hh form a regulatory loop in the embryonic epidermis and loss of Wg or Hh signalling leads to the loss of expression of all three genes, generating an identical phenotype at the end of embryogenesis. This characteristic segment polarity phenotype results from the simultaneous loss of polarity within each segment and the loss of naked cuticle (Fig. 1). Importantly, embryos without maternal and zygotic sgl, slf, ttv or frc exhibit this phenotype (Binari et al., 1997; Hacker et al., 1997; Haerry et al., 1997; Lin and Perrimon, 1999; The et al., 1999; Selva et al., 2001; Goto et al., 2001).

Also, RNA interference (RNAi) against dlp or daily generates embryos with weak segment polarity phenotypes (Lin and Perrimon, 1999; Tsuda et al., 1999; Baeg et al., 2001). Because embryos with weak segment polarity phenotypes (Lin and Perrimon, 1999; The et al., 1999; Selva et al., 2001; Goto et al., 2001), UASnuclacZ (Mark Muskavitch, Indiana University, USA), ftzlacZ (Jean Paul Vincent, Mill Hill, UK).

Materials and methods

Drosophila strains

The w1118 strain was used as wild type. wgCG4, hhAC and ptc1W (synonym of ptc1W) are null alleles (see FlyBase: http://gin.ebi.ac.uk:7081/).

We used the UAS/Gal4 system to drive ectopic expression (Brand and Perrimon, 1993). All overexpression experiments were carried out at 25°C. The transgenic strains used were: armGal411, armGal4-FRT-VP16 and KB19 (Sanson et al., 1996), simGal4 (Golembow, et al., 1996), UASwg (Lawrence et al., 1996), UASHh (Fietz et al., 1995), UASHh-N (Porter et al., 1996), UASdp (Baeg et al., 2001), UASnuclacZ (Mark Muskavitch, Indiana University, USA), ftzlacZ (Jean Paul Vincent, Mill Hill, UK).

Genotypes of embryos are as follow: armGal4; UASwg (Fig. 3B,C), simGal4; simGal4/UASwg (Fig. 3D-F), armGal4; UASHh (Fig. 4B,C), simGal4/UASnuclacZ; simGal4 (Fig. 4D), simGal4; simgal4/UAShh (Fig. 4E,F), armGal4; UASHh-N (Fig. 5A,C), UASHh-N; armGal4; hhAC/hhAC ftzlacZ (Fig. 5B) UASHh-N; simGal4; simGal4; simGal4 (Fig. 5D,F), UASHh-N; simGal4; hhAC/hhAC ftzlacZ (Fig. 5E), armGal4VP16/UASdp (Fig. 6A), enGal4; UASHh (Fig. 6B), enGal4; UASHh-N (Fig. 6C), ptc1W/ptc1W (Fig. 6D,E), ptc1W/CyOftzlacZ (Fig. 6F).

UASSh, UASHh-N (first and third chromosome insertions) and UASdp are homozygous stocks. UASwg is a heterozygous stock balanced over TM3hblacZ or TM3actGFP and lacZ or GFP embryo by ptc1W homozygous embryos layed by ptc1W/CyOftzlacZ flies were identified by the absence of lacZ expression.

RNA interference

For the synthesis of dsRNA, small regions of the plasmids pBS(KS)-dlp (Baeg et al., 2001) and pBS(KS)-dally (Nakato et al., 1995) were amplified by PCR with primers pairs containing a T7 promoter sequence at the 5’ end (5’TATACGACTCACTATAGG3’). The PCR products were used as templates for T7 transcription reactions with the Ribomax Large Scale Production kit (Roche). In these reactions, the two strands of RNA self-anneal. The dsRNA was extracted with phenol/chloroform, precipitated with ethanol and resuspended in injecting buffer. The concentration of dsRNA was evaluated on 1% agarose gel before and after extraction, and the volume of injection buffer adjusted to have a final concentration of about 5 µg/µL. Two dsRNA were prepared for silencing dlp (CG32146, NCBI accession noAE003554): 326 nucleotides (nt) from position 169 (primers 5’ACCAGTTGCACCTCAAC3’ and 5’CTGGAGTCGATGTGTC3’), 364nt from position 37772 (primers 5’AGAAATCTCCGCATCCAC3’ and 5’ATTTTGGATCCTGACATTGC3’). Three dsRNA were prepared for silencing daily (CG4974, NCBI accession noAE003553): 333nt from position 372 (primers 5’CTCTCTCTTCCGACCCAC3’ and 5’CAGACACAGTGATGATG3’), 394nt from position 59157 (primers 5’TGCAGTTTCACAGACCCAC3’ and 5’ATGGTGTGTGCAGATTG3’), and 30nt from position 61246 (primers 5’TAGCCACCGATATAACCG3’ and 5’GACTCCACTCTCTTGGGATG3’).

For injections, 2- to 4-day old synchronised flies were left to lay for 30 minutes at 25°C. Embryos were dechorionated in 50% commercial bleach and aligned on coverslips onto a strip of heptane glue. Embryos were then dissected, covered with Voltalief oil and injected at the posterior end. All these steps were done at 19°C and did not last more than 30 minutes to ensure that embryos were injected at early blastoderm, to minimise injection defects. After injection, embryos were examined under the dissecting microscope, and cellularised, unfertilised or damaged embryos were eliminated. w1118; injected embryos were left to develop at 18°C, whereas embryos carrying UAS/Gal4 transgenes were left to develop at 25°C. Cuticle preparations were standard (see below) but fixation of injected embryos for immunochemistry or in situ hybridisation was done as follow (Vincent and O’Farrell, 1992): Under the dissecting microscope, the Vortalef oil was removed as much as possible around the embryos using a razor blade. Embryos were then detached from the coverslip using heptane, transferred to a fixative solution of 10% formaldehyde in PBS, and left to fix for 35 minutes. The fixation was longer than standard protocols because of the presence of oil around the embryos. Fixed embryos were transferred to a Petri dish and devitellinised by hand using a sharp needle. The embryos were then dehydrated and stored in 100% methanol at –20°C.

RT-PCR

The RNAi efficiency was estimated by measuring endogenous mRNA levels using semiquantitative RT-PCR. Total RNA was isolated from batches of 60 injected embryos, after washing them with heptane to remove the Voltalief oil. The large number of embryos used in an extract was to ensure that the whole range of phenotypes observed with RNAI would be represented. Embryos were homogenised in 300 µl Trizol + 0.2 µg Linear Polyacrylamide as a carrier (Sigma),
followed by chloroform extraction and isopropanol precipitation. Extracted RNA was resuspended in 30 μl water, and 10 μl were used for reverse transcription, priming with oligo(dT). Reverse transcription reactions (20 μl) contained 10 μl RNA extract, 1 μl oligo(dT) 10 μM, 4 μl Superscript buffer, 2 μl DTT 0.1 M, 2 μl dNTPs 10 mM, 0.5 μl RNaseOut and 0.5 μl Superscript Reverse Transcriptase (all reagents are from Invitrogen, unless specified). After incubation for 1 hour at 42°C, the reactions were stopped by heating for 10 minutes at 65°C. PCR reactions (50 μl) used 1 μl of the reverse transcription reaction (which corresponds to 1 embryo equivalent of cDNA) and contained 5 μl 10x PCR buffer, 1 μl dNTPs 20 mM, TaqPlus polymerase 0.5 μl (Stratagene) and 2.5 μl of each primer (10 μM). Samples were denatured for 2 minutes at 94°C before cycling 30 times 1 minute at 55°C, 1 minute 30 seconds at 72°C and 30 seconds at 94°C. Primers used were: 5’ AGCAAAAAACATCCGCCGACG3’ and 5’ GCGATTGGAGCTGTTTGC3’ for dally (301nt product from positions 49658 to 50016), and 5’ ATTTGCGCGCCGAAACTG3’ and 5’ TGGCCATTGCTGTTTGC3’ for dally-like (305nt product from positions 37011 to 37314). 10 μl of each PCR reaction were resolved on 1% agarose gel stained with ethidium bromide, and quantification was done using the AlphaImager imaging system. Reactions with increasing numbers of cycles were initially run to determine the geometric phase: we could detect a geometric increase of PCR products between 25 and 35 cycles for both primer pairs, thus we used 30 cycles for subsequent RT-PCR reactions.

Embryos were fixed and stained according to standard protocols.

Results

RNAi silencing of dally-like but not dally generates a full segment polarity phenotype in the embryonic epidermis

To block dally or dally-like (dlp) function, we injected syncytial embryos with dsRNA fragments about 300 nucleotides long, which corresponded either to the 5’ or the 3’ end of the dlp mRNA or to the 5’, middle or 3’ of the dally mRNA (Fig. 1D). We allowed injected embryos to develop until the end of embryogenesis and examined their cuticle patterns (Fig. 1). A fraction (14%, n=99) of control embryos that were injected with buffer alone exhibited weak segmentation defects (Table 1 and not shown). These defects consist of fusions or deletions of denticles belts, which are distinct from transformation of naked cuticle to denticles seen in segment polarity mutants. They indicate a loss of tissue, and are presumably a consequence of the injection process. Injection of 3’, middle or 5’ dally dsRNA at a concentration of 5 μg/μl generates weak segmentation phenotypes identical to those of buffer-injected embryos, with the same frequency (15%, n=226) (Fig. 1F and Table 1). By contrast, injection of either the 3’ or 5’ dlp dsRNA, produces segmentation defects in almost all embryos, and about two-thirds of these exhibit either a strong or a full segment polarity phenotype. We scored as having a full segment polarity phenotype those embryos

Embryo preparations

For in situ hybridisation, embryo were fixed and hybridised with digoxigenin or fluorescein-labelled single stranded RNA probes as described by Jowett (Jowett, 1997), except that no proteinase K treatment was performed. wg, en and rho cDNAs were a gift from J. P. Vincent, and lacZ a gift from V. Morel.

Immunohistochemistry was done according to standard protocols. Primary antibodies used were mouse anti-En (1:50) (Hybridoma Bank) and rabbit anti-Dlp (1:50) (Baeg et al., 2001).

For cuticle preparations, embryos were mounted in Hoyer’s/lactic acid (1:1) and visualised with dark-field microscopy.

Fig. 1. RNAi silencing of dally-like but not dally generates a segment polarity phenotype in Drosophila embryos. (A) Ventral view of a wild-type larval cuticle. Unless specified, the head of embryos is to the left. Eight belts of denticles are visible in the abdomen. (B and C) Segment polarity phenotype of null mutations in wingless (wgCX4) and hedgehog (hhAc). The areas of naked cuticle are replaced by denticles, generating a lawn of denticles without clear polarity. (D) Position of the dsRNA sequences (in red) and the RT-PCR sequences (in blue) used in dally and dally-like RNAi experiments. (E) Agarose gel electrophoresis of semiquantitative RT-PCR reactions performed on extracts of embryos injected by either buffer, dlp 3’ dsRNA or dally 3’ dsRNA. There is at least a fourfold decrease in dlp mRNA following dlp RNAi, and at least a fivefold decrease in dally mRNA following dally RNAi. By contrast mRNA levels of dally and dlp following dlp and dally RNAi, respectively, are identical to the levels after injection of buffer. (F) Weak segmentation defects exhibited by dally dsRNA-injected embryos. The same type of defects are found in buffer-injected embryos (not shown), and thus are a consequence of the injection process. There is no clear transformation of naked cuticle into denticles in these embryos, but rather a loss of tissue that leads to the fusion of denticles belts. (G-I) Segment polarity phenotypes found in dlp dsRNA injected embryos. (G) The weak segment polarity phenotypes of dally-like and (I) lawn of denticles identical to the phenotype of wingless or hedgehog null mutants. (See also Table 1.)
Embryos were injected with either buffer, dally or dally-like dsRNA, and the cuticle of the ventral abdomen scored for segmentation defects at the end of embryogenesis. Only embryos with an intact cuticle in the trunk were analysed (n=total number of analysed embryos). Segmentation defects were classified into three categories. In ‘lawn of denticles’ and ‘strong segmentation defects’, every segment had transformations of naked cuticle into denticles, with some naked cuticle remaining in embryos of the ‘strong segmentation defects’ category. The other embryos with segmentation defects were categorised as weak. Only weak segmentation defects were found after injection of dally dsRNA or buffer. dally RNAi was performed using the 5’ and 3’ dsRNA sequences. The same proportion of weak segmentation defects was observed in each case and the results have been added together for this table. For dally-like, injections were performed with a 5’ dsRNA or a 3’ dsRNA. The same distribution of weak, strong and lawn phenotypes were observed (not shown). The table shows the result of a typical experiment using the 5’ dsRNA sequence, which is the dsRNA used for the RNAi experiments presented in Figs 2-6.

Table 1. Distribution of the segmentation defects in embryos injected with dally or dally-like dsRNA

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Segmentation defects (total)</th>
<th>Weak segmentation defects</th>
<th>Strong segmentation defects</th>
<th>Lawn of denticles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>99 (14%)</td>
<td>14 (14%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>dally RNAi</td>
<td>226 (35%)</td>
<td>35 (15%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>dally-like RNAi</td>
<td>211 (193 (91%)</td>
<td>60 (28%)</td>
<td>46 (22%)</td>
<td>87 (41%)</td>
</tr>
</tbody>
</table>

Embryos were injected with either buffer, dally or dally-like dsRNA, and the cuticle of the ventral abdomen scored for segmentation defects at the end of embryogenesis. Only embryos with an intact cuticle in the trunk were analysed (n=total number of analysed embryos). Segmentation defects were classified into three categories. In ‘lawn of denticles’ and ‘strong segmentation defects’, every segment had transformations of naked cuticle into denticles, with some naked cuticle remaining in embryos of the ‘strong segmentation defects’ category. The other embryos with segmentation defects were categorised as weak. Only weak segmentation defects were found after injection of dally dsRNA or buffer. dally RNAi was performed using the 5’, middle or 3’ dsRNA sequences. The same proportion of weak segmentation defects was observed in each case and the results have been added together for this table. For dally-like, injections were performed with a 5’ dsRNA or a 3’ dsRNA. The same distribution of weak, strong and lawn phenotypes were observed (not shown). The table shows the result of a typical experiment using the 5’ dsRNA sequence, which is the dsRNA used for the RNAi experiments presented in Figs 2-6.

showing a lawn of denticles ventrally, with no naked cuticle left (Fig. II). Embryos with all segments affected, but with naked cuticle patches remaining were scored as strong segment polarity phenotypes (Fig. IH). The rest of the embryos were classified as having weak segmentation defects (Fig. IGI), although most of these embryos showed stronger defects than in dally dsRNA injection or buffer injection (Fig. IF and not shown). Table 1 shows the distribution of phenotypes for a typical experiment using the dlp 3’ dsRNA sequence: 91% of injected embryos showed segmentation defects, and 63% exhibited a strong or full segment polarity phenotype (n=211). Both 3’ and 5’ sequences gave the same distribution of phenotypes (data not shown).

To check the efficiency of the RNAi, we used semiquantitative RT-PCR to monitor the levels of dally or dlp mRNAs in injected embryos. Total RNA was extracted from batches of 60 injected embryos after 7 hours of development (stage 11/12), and the derived cDNAs were amplified by PCR using oligonucleotide pairs targeting a 300 nt sequence just upstream of the 3’ dsRNA sequences (Fig. 1D). RT-PCR on extracts from buffer-injected embryos detected similar mRNA levels for dally and dlp (Fig. 1E). RT-PCR on extracts from embryos injected with the 3’ dsRNA sequence of dally showed a strong reduction of dally mRNA as expected, while dlp mRNA levels were unchanged (Fig. 1E). The converse is true for RT-PCR on extracts of embryos injected with the 3’ dsRNA sequence of dlp; dlp, but not dally, mRNA levels were strongly reduced (Fig. 1E). This demonstrates that RNAi efficiently silences both dally and dlp, and also that RNAi directed at one gene does not affect the other.

These results show that RNAi silencing of dlp produces a severe and penetrant segment polarity phenotype. Since dlp mRNA is produced maternally as well as zygotically (Khare and Baumgartner, 2000), the strength of the phenotype suggests that injection of dsRNA inactivates both pools of mRNA. The full phenotype is identical to wg or hh loss-of-function phenotypes in embryos (compare Fig. II with B,C). Previous work reported weaker segment polarity phenotypes in the cuticle following dlp RNAi (Baeg et al., 2001). A possibility is that the use of small dsRNA sequences (300-400nt) in our study, rather than dsRNA corresponding to larger parts of the gene, allowed us to inject higher concentrations of dsRNA, and as a consequence to obtain stronger phenotypes.

In contrast to previous reports (Lin and Perrimon, 1999; Tsuda et al., 1999; Baeg et al., 2001), we did not obtain any segment polarity phenotypes with dally RNAi, despite testing three different dsRNA sequences and using a high concentration of dsRNA. Furthermore, the RT-PCR controls show that dally had been efficiently silenced in our experiments. A possible explanation for this discrepancy is that, in previous studies, the use of a dsRNA corresponding to large sequences of dally had resulted in partial silencing of dlp, because of small regions of sequence homology.

dally-like RNAi mimics hedgehog loss of function in the embryonic epidermis

All mutations that give strong segment polarity phenotypes disrupt either Wg or Hh signalling, leading to a loss of en expression at mid-embryogenesis. Wg is required to maintain the expression of en across the parasegmental boundary, whereas Hh is secreted by the en cells, and in turn maintains wg expression (Fig. 2E) (Martinez Arias, 1993). In wg null mutant embryos, En protein disappears completely from the ectoderm of the trunk by stage 11 (Fig. 2B). In a hh null mutant, En starts disappearing at stage 11 and is mostly gone at stage 12 (Fig. 2C,H). In dlp RNAi embryos, En starts to be lost at stage 11, and by late stage 12 only patches of En remain in the ectoderm (Fig. 2D,I). The timing of the loss of En resembles that of a hh null mutant rather than a wg null mutant. In contrast, dally RNAi embryos did not show any defects in the pattern of En (data not shown).

We also looked at the pattern of rhomboid (rho) transcription in RNAi dlp embryos. In wild-type embryos, rho is expressed in a single stripe posterior to the en domain (Fig. 2K), because it is activated by Hh and Serrate (Ser) signalling and repressed by Wg signalling (Alexandre et al., 1999) (Fig. 2O). In wg mutants, two stripes of rho expression were found on both sides of the cells that were expressing en (Fig. 2L). In contrast, only one stripe of rho expression was found in hh mutants as in wild type, presumably because there is enough Wg activity left to repress the anterior stripe, while part of the posterior stripe was maintained by Ser signalling (Fig. 2M). In embryos injected for dlp dsRNA, we found only one stripe of rho expression, as in hh mutants (Fig. 2N). Taken together, these results suggest that loss of dlp mimics hh loss of function rather than wg loss of function.

RNAi silencing of dlp and dally do not inhibit Wg signalling

To uncouple the regulatory loop between Wg and Hh signalling in embryos, we expressed UASwg under the control of two Gal4 drivers, armadillo-Gal4 (armGal4) and single-minded-Gal4 (simGal4), and looked at two different targets of Wg signalling: the transcription of en and the specification of naked cuticle. ArmGal4 drives expression ubiquitously (Sanson et al., 1996), and simGal4 drives expression in the ventral midline (Golembo et al., 1996). As Wg specifies naked cuticle in most
segments through the repression of the zinc-finger transcription factor shavenbaby (Payre et al., 1999), the secretion of naked cuticle gives a convenient read-out of Wg signalling activity (Lawrence et al., 1996; Sanson et al., 1999).

In armGal4/UASwg embryos, en transcription was enlarged from a stripe about two cells wide to a stripe spanning about half a segment, which coincided with the domain of competence for en transcription (Fig. 3A,B). In armGal4/UASwg [dlp RNAi] embryos, en ectopic expression is unaffected (Fig. 3C). Expression using simGal4 allows the examination of non cell-autonomous signalling, because Wg is secreted by the midline cells and acts on the adjoining epidermal cells. In simGal4/UASwg embryos, cells specified naked cuticle in the ectoderm a few cell diameters away on either sides of the midline (Fig. 3D). We could not look at en transcription in this experiment because simGal4 expression starts at stage 11, when en expression has become independent of Wg signalling. In simGal4/UASwg [dlp RNAi] embryos, naked cuticle specification was unaffected around the midline (Fig. 3E). The epidermis also exhibited a strong segment polarity phenotype lateral to the stripe of naked cuticle, demonstrating that dlp has been silenced efficiently and thus providing an internal control for the experiment. Together, the simGal4 and armGal4 experiments suggest that Dlp is not required for Wg signalling in embryos.

It is possible that Dally and Dlp function redundantly in Wg signalling in embryos. To test this, we injected dally 3' dsRNA into simGal4/UASwg embryos, and found that no segment polarity phenotype could be detected in the lateral domain, as expected, and that the formation of naked cuticle was unaffected at the midline (not shown). These experiments suggest that dally and dlp are dispensable for Wg signalling in embryos, and that they do not function redundantly in this pathway.

**Dally-like is required for Hedgehog signalling**

Since dlp RNAi gives a strong segment polarity phenotype but does not affect Wg signalling, it is probable that Dlp is required for either Hh transcription or Hh signalling. To test this, we expressed Hh ubiquitously with armGal4, and used the activation of wg transcription as a read-out of Hh signalling. In armGal4/UAShh embryos, wg expression was enlarged from a stripe one cell wide to a stripe covering about half a segment, which corresponded to the domain of competence for wg transcription (Fig. 4A,B). In armGal4/UAShh embryos injected with dlp dsRNA, both ectopic and endogenous wg expression were lost at stage 11 (Fig. 4C). We also looked at the non cell-autonomous effect of Hh, expressing UAShh under the control of simGal4 (Fig. 4D). In simGal4/UAShh embryos, Hh activated the transcription of wg in the ectoderm, a few cell diameters on either side of the midline, within the wg competence domain of each segment (Fig. 4E). In simGal4/UAShh [dlp RNAi] embryos, both endogenous and ectopic wg expression were lost at stage 11 (Fig. 4F). Since dlp RNAi does not affect the activity of the armGal4 and simGal4 drivers (see previous section), these experiments demonstrate that Dlp is required for Hh signalling.
Dally-like is required downstream of Hedgehog processing

Dlp could be required either for Hh secretion from the signalling cells, for Hh movement, or for Hh signal transduction. In the secreting cells, Hh undergoes an intramolecular cleavage which is catalysed by the C terminus of the protein. The liberated N-terminal fragment (the active ligand) is coupled to a cholesterol moiety (Ingham and McMahon, 2001). To determine whether dlp is required in the signalling cells, we expressed an engineered form of Hh, Hh-N, which is pre-cleaved and not modified by cholesterol (Porter et al., 1995) and tested whether Hh-N could bypass the requirement for dlp. ArmGal4/UASHh-N embryos ectopically expressed wg in the same pattern as armGal4/UASHh embryos (compare Fig. 4B and Fig. 5A). SimGal4/UASHh-N embryos ectopically expressed wg on both sides of the midline, as in simGal4/UASHh embryos, but over a longer distance from the midline (compare Fig. 4E and Fig. 5D). This is consistent with previous findings that Hh-N moves further in a field of cells than Hh (Burke et al., 1999; Porter et al., 1996). In both armGal4/UASHh-N and simGal4/UASHh-N embryos injected with dlp dsRNA, both ectopic and endogenous wg expression are gone (97%, n=29), confirming the requirement of dlp for Hh signalling (F). All embryos shown are late stage 11.
patches of wg ectopic expression remained in simGal4/UASSh-N[hh-] embryos (Fig. 5E). This indicates that Hh-N can still signal in the absence of endogenous Hh, but that Hh-N might be dependent upon Hh for its movement from cell to cell. In contrast, all wg expression was lost in armGal4 or simGal4/UASSh-N[dlp RNAi] embryos (Fig. 5C,F), showing that the activity of Hh-N is absolutely dependent on Dlp. We conclude that Dlp is required downstream of Hh processing and cholesterol modification.

**Increasing the concentration of Dally-like does not increase the range of Hedgehog signalling**

To test if Dlp facilitates the movement of Hh in the ectoderm, we overexpressed Dlp in embryos, and assessed the range of Hh signalling by looking at the width of the wg stripe (Fig. 6A). Overexpressing UASdlp with either enGal4 or ptcGal4 did not have any effect on wg expression and the embryos were viable (data not shown). As a control, we immunostained for the Dlp protein in enGal4/UASdlp embryos, and found Dlp expressed in stripes as expected (not shown). Also, enGal4/UASdlp adult flies exhibited notched wings as previously observed (Baeg et al., 2001) and ptcGal4/UASdlp flies died at the pharate stage (data not shown), thus indicating that UASdlp expressed an active protein. To increase the quantity of Dlp protein made, we used the armGal4VP16 driver, which is the strongest driver available in embryos (Sanson et al., 1996). We could not detect any enlargement of the wg stripe in armGal4VP16/UASdlp embryos (Fig. 6A). As a comparison, expressing UASSh in en cells did lead to a moderate but detectable enlargement of the wg stripe at stage 11 (Porter et al., 1996) (Fig. 6B). Furthermore, overexpression of UASSh-N in the same cells lead to a larger stripe of wg expression, consistent with the idea that Hh-N can travel a longer distance than Hh (Porter et al., 1996) (Fig. 6C). We conclude that increasing the concentration of Dlp in embryos does not increase the range of Hh signalling.

**Dally-like is required upstream or at the level of the Patched receptor**

Since Dlp is not required for Hh processing and cholesterol modification, and does not stimulate Hh movement when overexpressed, it is probable that Dlp is required for the transduction of the signal. In absence of the Patched (Ptc) receptor, Smoothened constitutively activates the Hh intracellular pathway (Ingham and McMahon, 2001). We looked at this constitutive signalling in ptc mutants, in the presence or absence of dlp, using wg transcription as a read-out. In embryos homozygous for the null mutation ptc IIW, wg transcription was enlarged in a pattern similar to that in armGal4/UASsh embryos (Fig. 6D). When we injected dlp dsRNA into ptc IIW embryos, most of the homozygous embryos maintained wg ectopic expression (Fig. 6E). Three homozygous embryos out of thirty (10%) had partially lost wg expression, but close examination showed that loss of expression was always associated with a disrupted epithelium. Thus, in ptc- embryos, injection of dlp dsRNA does not abolish Hh constitutive signalling. In contrast, wg endogenous expression disappeared in 78% of the heterozygous embryos (n=58), showing that dlp has been efficiently silenced in this experiment (Fig. 6F). We conclude that ptc is epistatic to dlp. This indicates that Dlp acts either upstream or at the level of the Ptc receptor.

**Discussion**

Heparan sulphate modifications have been implicated in several signalling pathways during animal development, including the Hh and Wg signalling pathways, based on the phenotype of mutants in enzymes required for heparan sulphate biosynthesis. However, it remains unclear which proteins are modified by these enzymes, and how the modifications affect a given signalling event. Since most heparan sulphate chains at the cell surface are thought to be carried by proteoglycans
of the syndecan or glypican families, we have examined the function of the two Drosophila homologues of glypicans, daily and daily-like (dlp), in the embryonic epidermis. Both glypicans had been suggested previously to play a role in the Wg signalling pathway. Unexpectedly, we found a much more restricted and specific role for the fly glypicans. We have shown by RNAi silencing that Dlp is a segment polarity gene that is absolutely required for Hh signalling. This requirement is specific to the Hh pathway, as we show that RNAi silencing of dlp does not affect Wg signalling in embryos. In contrast, RNAi silencing of daily, the other homologue of glypicans in Drosophila, does not produce a segment polarity phenotype, suggesting that Daily is dispensable for Wg or Hh signalling in embryos. Furthermore, RNAi silencing of both daily and dlp does not affect Wg signalling, suggesting that they do not function redundantly in this pathway.

**Role of Dally-like in the Hedgehog pathway**

dlp is a bona fide segment polarity gene since dlp RNAi generates embryos that fail to maintain en and wg expression at mid-embryogenesis, and exhibit a full segment polarity phenotype in the cuticle at the end of embryogenesis (Fig. 1, 2 and not shown). The late disappearance of en expression and the single stripe of rho expression in dlp embryos suggest a loss of Hh activity (Fig. 2). This is confirmed by the fact that when hh expression is under heterologous control, ectopic wg transcription is lost in dlp RNAi embryos, whether Hh is provided autonomously (armGal4 experiments) or non-autonomously (simGal4 experiments) (Fig. 4). These experiments demonstrate unambiguously that dlp is required for Hh signalling and rule out a requirement for hh transcription.

Dlp is a GPI-anchored protein and is likely to be localised at the cell surface. This leaves two plausible roles for Dlp: either it is required for the release of active Hh from the secreting cells, or it is required for the interpretation of the Hh signal on the receiving cells. Our experiments eliminate several possibilities. First, Dlp is required for the activity of Hh-N, an engineered form of Hh which is pre-processed and unmodified by cholesterol (Fig. 5). This suggests that Dlp is necessary downstream of Hh processing and cholesterol modification. Downstream of these events, Hh undergoes another lipid modification, the addition of a palmitoyl moiety. The segment polarity gene rasp codes for an acyltransferase which is thought to be needed for Hh palmitoylation (Amanai and Jiang, 2001; Chamoun et al., 2001; Lee and Treisman, 2001; Micchelli et al., 2002). Thus, Dlp could be required for the function of rasp in the signalling cells. However, whereas palmitoylation is essential for Hh-N activity, a recent report shows that it is not strictly required for the activity of wild-type Hh in Drosophila embryos (Gallet et al., 2003). This suggests that the cholesterol and palmitoylate modifications might be partially redundant for the activity of wild-type Hh, at least in embryos. Thus, although Dlp could still act at the level of rasp on another function, loss of palmitoylation alone cannot account for the complete loss of Hh signalling seen in dlp RNAi embryos. It seems therefore more likely that dlp functions in the responding cells.

We show that ptc is epistatic to dlp, indicating that Dlp acts upstream or at the level of the Ptc receptor (Fig. 6D-F). One possibility is that Dlp binds Hh and facilitates its interaction with Ptc. Increasing the concentration of Hh in receiving cells in either armGal4/UAShh or armGal4/UAShh-N experiments, does not abolish the requirement for Dlp. This argues against a role of Dlp in merely increasing the concentration of Hh ligand at the cell surface, and suggests a more specific role. Recent evidence supports a model in which, upon Hh binding, Ptc is endocytosed and inactivated by degradation, and this in turn indirectly activates Smoothened and the Hh intracellular pathway (Denef et al., 2000; Martin et al., 2001; Strutt et al., 2001). Dlp may localise Hh and Ptc in membrane microdomains required for Ptc endocytosis and subsequent degradation.

While we were completing this manuscript, Lum and
Dlp is required for Hh signalling in *Drosophila*

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colleagues (Lum et al., 2003) reported that Dlp is required for the transduction of the Hh signal in cultured *Drosophila* cells, using RNAi silencing. The design of the experiment eliminates the requirement for Hh secretion or distribution, showing that Dlp is required for the reception of the signal in cultured cells. In agreement with our results, Lum and colleagues find that the requirement for Dlp in Hh signal transduction is suppressed by expressing Hh in responding cells. We did not find this in either armGal4/UAShh or armGal4/UAShh-N experiments. This difference may be due to differences in dose: transfection of Hh in cultured cells may generate protein levels well above physiological levels, whereas Gal4 expression levels are in the range of endogenous expression levels. Another difference is that overexpression of Dlp in cultured cells stimulates the response to Hh, in a manner comparable to overexpression of Cubitus interruptus (Ci), the downstream component of the Hh pathway. Using UASDlp and several Gal4 drivers including armGal4VP16, we were not able to detect any stimulation of wg transcription in embryos following Dlp overexpression (Fig. 6A). In contrast, overexpression of Ci in embryos does stimulate wg transcription (Alexandre et al., 1996) (data not shown). A possibility is that endogenous *dlp* is expressed at low levels in cultured cells and is therefore limiting, but is not limiting in embryos.

**Drosophila glypicans and Wingless signalling**

We did not find a requirement for the fly glypicans Dlp and Dally in Wg signalling in the embryonic epidermis. In agreement with our findings in vivo, Lum and colleagues showed that RNAi of *daily* or *dlp* does not affect Wg signalling in a cell culture assay (Lum et al., 2003). We found that when Wg expression is under heterologous control (using armGal4 or simGal4), and thus independent of Hh signalling, en maintenance and naked cuticle secretion are normal in *dlp* RNAi embryos (Fig. 3). Since these two events are under direct control by Wg in the ventral epidermis, this suggests that Dlp is not necessary for Wg signalling in embryos. RNAi silencing of *daily*, using three different dsRNA sequences and high concentration of dsRNA, did not give any segment polarity phenotype in the cuticle and did not affect the pattern of *en* expression or the secretion of naked cuticle (Table 1, Fig. 1 and 3, not shown). Our RT-PCR experiment demonstrates that *daily* RNAi has worked and leads to a strong reduction in *daily* mRNA levels (Fig. 1E). This result is in contrast with previous reports showing weak segment polarity phenotypes following *daily* RNAi (Lin and Perrimon, 1999; Tsuda et al., 1999; Baeg et al., 2001). It is possible that the use of dsRNA corresponding to larger parts of the *daily* sequence, in these earlier reports, might have resulted in partial silencing of *dlp* through short stretches of sequence homology. To avoid this potential problem, we used short sequences (300–400 nt), and chose sequences with the least homology between *daily* and *dlp* or any other sequences in the genome. Our RT-PCR experiments confirm that RNAi silencing of *daily* did not affect the mRNA levels of *dlp* and vice versa (Fig. 1E).

A possible explanation for an absence of effect in Wg signalling after *daily* or *dlp* RNAi is that the two genes function redundantly in Wg signalling in embryos, as it is the case for the Wg receptors Frizzled and Frizzled2 (Wodarz and Nusse, 1998). We tested this hypothesis by co-injecting *daily* and *dlp* dsRNA in simGal4/UASwg embryos, and show that ectopic Wg signalling is unaffected (Fig. 3F). Importantly, the simGal4 experiments effectively mimic the production of ligand in the wild type because (1) the UAS transgene is expressed in a thin stripe, which allows the monitoring of non-cell autonomous signalling, and (2) the UAS transgene is expressed at levels similar to endogenous transcription (see Fig. 4D). In conclusion, our work suggests strongly that Dally and Dlp, separately or together, are not necessary for Wg signalling in embryos. It has to be noted, however, that if RNAi decreases dramatically the amount of zygotic and maternal mRNAs in embryos, it does not affect the maternal protein stores, and thus we cannot formally rule out that some maternal Dally and/or Dlp protein are sufficient for rescuing Wg signalling in our experiments. Germline clones of null mutations in *daily* and *dlp*, to remove maternal and zygotic contribution of both genes, will need to be performed to definitively settle this issue.

Dally and Dlp could affect Wg distribution or movement without being required for Wg signalling in embryos. We could detect a slight increase in the extent of naked cuticle secreted in enGal4/UASwg [daily RNAi] embryos, which is the assay we use to look at the range of Wg (S.D. and B.S., unpublished). Lum and colleagues also mentioned that Wg distribution at the cell surface is changed in cultured cells silenced for *daily* by RNAi (Lum et al., 2003). This is compatible with an earlier suggestion that HSPPs are needed for the retention of Wg at the surface and within the secretory pathway of expressing cells (Pfeiffer et al., 2002). So removal of Dally might affect Wg distribution in embryos or cell culture, but without detectable impact on Wg signalling. In the wing disc, overexpression of Dlp, but not Dally, has been shown to stabilise Wg at the cell surface (Baeg et al., 2001; Strigini and Cohen, 2000). It has been hypothesised that Notum, which has similarities with pectin acetylesterases, could modify the affinity of Dally and Dlp for Wg (Gerlitz and Basler, 2002; Giraldez et al., 2002). Thus different modifications of the glypicans by tissue-specific enzymes such as Notum, could account for the differences between the embryo and the wing disc. However, it has not yet been proved that there is a direct interaction between Wg and Dally and Dlp, and if this interaction has a biological significance.

**Drosophila glypicans and glycosaminoglycan modifications**

So far, four genes coding for enzymes necessary for heparan sulphate biosynthesis give a segment polarity phenotype in *Drosophila* embryos: *sugarless* (*sgl*), *sulfateless* (*sfl*), *tout-velu* (*ttv*) and *fringe connection* (*frc*). The segment polarity phenotypes are seen after removal of both maternal and zygotic contribution of any of these genes and are identical to *wg* or *hh* null mutant phenotypes (Binari et al., 1997; Hacker et al., 1997; Haerry et al., 1997; Lin and Perrimon, 1999; The et al., 1999; Selva et al., 2001; Goto et al., 2001). *sgl*, *sfl*, *ttv* and *frc* have similarities with vertebrate genes coding for a UDP-glucose dehydrogenase, a N-deacetylase/N-sulphotransferase, a heparan sulphate co-polymerase (Ext1), and a UDP sugar transporter, respectively. Consistent with this, mutations in all
four genes affect glycosaminoglycan biosynthesis: Sgl affects both chondroitin and heparan sulphate synthesis, Sff reduces the proportion of sulphated disaccharides in heparan sulphate, Ttv dramatically decreases all forms of heparan sulphate and Frc reduces the amount of heparan sulphate in embryos (Toyoda et al., 2000; The et al., 1999; Selva et al., 2001). Since glypicans carry heparan sulphate chains, they have been hypothesised to be the target of these enzymes. However, sff and sgl have both been implicated in Wg signalling but not Hh signalling in embryos (Binari et al., 1997; Hacker et al., 1997; Haerry et al., 1997; Lin and Perrimon, 1999). The fact that neither dally nor dlp seem to be required for Wg signalling in embryos (our study) or in cultured cells (Lum et al., 2003) poses a paradox. A possibility is that dally and dlp are solely required for Wg distribution and/or transport, as opposed to Wg signal transduction, and that this requirement is not revealed after RNAi in embryos or in cells. Alternatively, Sff and Sgl might be required for the function of another heparan sulphate proteoglycan or for the glycosylation of a protein with a central role in Wg signalling. The fourth gene, tvt, was found to act specifically in the Hh signalling pathway, and thus could exhibit a segment polarity phenotype because of loss of Dlp activity. However, Ttv is required for Hh movement in wing discs, but not for Hh signalling per se (Bellaiche et al., 1998; The et al., 1999). Dlp is strictly required for Hh signalling but does not seem to influence its movement in embryos, at least in an overexpression assay (Fig. 6A). Furthermore, RNAi silencing of tvt and its two Drosophila homologues Exr2 and Exr3, individually or in combination, does not inhibit Hh signalling in cell culture (Lum et al., 2003). Thus, the basis for the segment polarity phenotype of tvt mutants remains to be clarified.

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References


Dlp is required for Hh signalling in Drosophila


