Hedgehog Signal Transduction in the Posterior Compartment of the Drosophila Wing Imaginal Disc


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Summary

Drosophila Hedgehog (Hh) is secreted by Posterior (P) compartment cells and induces Anterior (A) cells to form a developmental organizer at the AP compartment border. Hh signaling converts Fused (Fu) to a hyperphosphorylated form, Fu*. We show that A border cells of wing imaginal discs contain Fu*. Unexpectedly, P cells also produce Fu*, in a Hh-dependent and Ptc-independent manner. Increasing Ptc, the putative Hh receptor expressed specifically by A cells, reduced Fu*. These results are consistent with proposals that Ptc downregulates Hh signaling and suggest that a receptor other than Ptc mediates Hh signaling in P cells of imaginal discs. We conclude that Hh signals in these P cells and that the outputs of the pathway are blocked by transcriptional repression.

Introduction

The Hedgehog (Hh) signaling pathway has an instructive role in the development of many vertebrate and insect organs. In Drosophila imaginal discs, Hh produced by Posterior (P) compartment cells induces Anterior (A) cells to form a developmental organizer (Basler and Struhl, 1994; Tabata and Kornberg, 1994). This effect is limited to a strip of cells adjacent to the A/P compartment border, where Hh signals A cells to upregulate expression of target genes such as decapentaplegic (dpp) and patched (ptc) (reviewed in Ingham, 1998).

Ptc is a protein with multiple putative membrane-spanning domains. It is expressed in all A cells, and it functions to limit the influence of Hh to a few cells on the A side of the compartment border. Genetic interactions between hh and ptc that indicate that Ptc functions downstream of Hh in cells that receive the Hh signal led to the proposal that Ptc is the Hh receptor (Ingham et al., 1991). This view is supported by the demonstration that vertebrate Ptc binds Sonic Hh (Marigo et al., 1996; Stone et al., 1996), although no direct evidence that Drosophila Hh binds to Ptc has been reported. Another candidate for the Hh receptor is Smoothened (Smo), a protein with seven putative membrane-spanning domains that is a member of the family of proteins that includes the Wingless receptors (Alcedo et al., 1996; van den Heuvel and Ingham, 1996). Genetic interactions indicate that smo is downstream of both ptc and hh.

Since the smo and hh mutant phenotypes are similar and ptc;hh double mutants have a phenotype like ptc (Ingham et al., 1991; Bejsovec and Wieschaus, 1993; Hooper, 1994), a model for Hh signaling has been proposed whereby Ptc negatively regulates Smo, except when bound by Hh, and Smo signals constitutively except when inhibited by Ptc (reviewed in Ingham, 1998).

The response triggered by the Hh receptor involves in whole or in part the transformation of a microtubule-bound cytoplasmic complex. A cells in the trunk of the embryo and in imaginal discs have a protein complex that includes Cubitus interruptus (Ci), a transcription factor, Fused (Fu), a putative serine/threonine protein kinase that is altered by Hh signal transduction, and Costal-2, a kinesin-related microtubule binding protein (Robbins et al., 1997; Sisson et al., 1997). Ci is converted to a transcriptional activator (Aza-Blanc et al., 1997; Ohlmeyer and Kalderon, 1996; Méthot and Basler, 1999), the phosphorylation of Ci is altered (Chen et al., 1999; Price and Kalderon, 1999), and the limited proteolysis that converts Ci to a transcriptional repressor is inhibited (Aza-Blanc et al., 1997). In addition, the association between the complex and microtubules is weakened (Robbins et al., 1997), and Fu is converted to a hyperphosphorylated state (Fu*) (Thérond et al., 1996). Neither the pathway that leads to these changes nor the role of Fu (or Fu*) is known.

Several observations cannot be easily reconciled with this pathway of Hh signal transduction or with the model of a complex Hh receptor consisting of Ptc and Smo. Certain neuroblasts in the Drosophila embryo whose maturation is dependent on hh do not express or require ptc (Bhat and Schedl, 1997). In addition, development of Bolwig’s organ requires hh and ptc but not ci or fu (Suzuki and Saigo, 2000). These observations suggest that Ptc may not be the only Hh receptor and that the Hh pathway may signal through different components in different organs or cells. To explore the mechanism of Hh signaling further, we monitored Fu* in various cell types. We found that Hh activates signal transduction in both P and A cells of wing imaginal discs, despite the absence of Ptc in P cells. Since P cells do not express Hh target genes, this observation suggests that P cells regulate their response to Hh with a novel mechanism—by making their Hh target genes insensitive to Hh signaling.

Results

hh, smo, and ptc Regulate Fu Phosphorylation in Drosophila Embryos

To study the conversion of Fu to Fu*, we analyzed immunoblots of extracts prepared from wild-type and mutant embryos and imaginal discs. Since the hh, smo, and ptc mutants are embryonic lethal and cannot be distinguished by morphology until after Hh signaling has begun, we developed a set of modified balancer chromosomes to distinguish mutant embryos at an early stage of embryogenesis. These balancer chromosomes express Green Fluorescent Protein (GFP; Casso et al., 1999), and in a cross of balanced heterozygous parents, mutant embryos can be distinguished by their lack of...
mutant embryos could be generated either by Hh signaling or by a Smo-dependent process that is independent of Hh. To distinguish between these possibilities, we analyzed the state of Fu phosphorylation in ptc;hh double mutants. Two different ptc null alleles were examined: ptc1, which is a protein null mutant and ptcCE, which is a ptc gene deletion. The hh allele, hhCE, is a gene deletion. In both types of double mutants, Fu* was present in abundance and its level and distribution were indistinguishable from ptc single mutants (Figure 1C; data not shown). The presence of Fu* in ptc;hh double mutants indicates that in the absence of Ptc, Fu* is produced independently of Hh and that Fu phosphorylation is not coupled to Hh function.

A Border Cells and P Cells Respond to Hh

The prevailing model for Hh signaling assumes that in wild-type animals only those A cells near the A/P compartment border activate the Hh signal transduction pathway. This model is based on the observation that Fu phosphorylation is not coupled to Hh function. However, this explanation assumes that no Hh activity remains in the mutant embryos, and since Ptc negatively regulates the Hh pathway, even a low level of Hh might affect ptc mutants significantly. We verified that the embryo phenotype of the protein null ptc mutants, ptcCE and ptc5, are not altered by deletion of hh (Figures 2A-2D; data not shown). However, Hh is produced in the germline (Forbes et al., 1996; Chen and Baker, 1997; Gorfinkel et al., 1999), and it is conceivable that Ptc, which is expressed broadly in young embryos (Hooper and Scott, 1989; Nakano et al., 1989) might suppress any Hh produced by hh RNA that remains after fertilization. To investigate whether ptc mutants have residual Hh due to their inability to suppress maternal Hh, double-stranded hh RNA was injected into wild type and ptc mutant embryos. We found that hh RNA can approximate a hh null condition in normal embryos (Figure 2E), but that it did not alter the ptc phenotype (Figure 2F). We verified that RNAi injections can phenocopy maternal-effect mutants by injecting smo RNAi. Cuticle patterns produced by injected normal or ptc5/ptcCE embryos were indistinguishable from smo- embryos derived from smo germline clones (Figures 2I and 2J). This result is consistent with previous analyses of smo;ptc zygotic mutants (Alcedo et al., 1996), and indicates that smo function is needed to generate the ptc phenotype.

As a further test, the role of Ci in the ptc;hh phenotype was assessed. The transcriptional output of the Hh signal transduction pathway is mediated through Ci, so if the absence of Ptc results in activation of the Hh signaling pathway, the expectation would be that Ci function is needed to generate the ptc;hh phenotype. We tested this by injecting ci RNAi into normal and ptc mutant embryos. A cuticular conversion to a ci- phenotype was observed in both types of embryos (Figures 2G and 2H), consistent with the proposal that the Hh signal transduction pathway is activated in ptc- embryos.
pathway and predicts that Fu* should be confined to these A border cells. We developed methods to isolate geographically distinct populations of cells from wing imaginal discs and subjected these cells to assays for Fu*. Three populations of cells were isolated: A and P cells, by dissection; and A cells that populate the A/P compartment border region by flow cytometry (see the Experimental Procedures). Western analysis revealed that the cells in A fragments contain Fu, that the cells in P fragments contain Fu*, and that A border cells contain a mixture of both forms of Fu (Figure 1D). These results confirm that A cells near the compartment border respond to Hh signaling and that cells elsewhere in the A compartment do not. This pattern of Hh signaling in the A compartment is consistent with previous proposals that Hh signaling is limited to cells near the compartment border (Tabata and Komberg, 1994; Chen and Struhl, 1996). The presence of Fu* in the P compartment was unexpected and indicates that Hh-producing cells are not refractory to Hh as had been previously postulated (Zecca et al., 1995). Moreover, since P cells do not express ptc (Hooper and Scott, 1989; Nakano et al., 1989), Hh signal transduction in P cells is apparently Ptc independent.

To further characterize Hh signal transduction in P cells, we examined the phosphorylation state of Fu in imaginal discs that lack Hh function. Wing imaginal discs homozygous for a hh<sup>ts</sup> allele were incubated at either permissive or nonpermissive temperatures, and extracts were prepared. At the permissive temperature, the proportion of Fu and Fu* was 1:1 (Figure 1E). However, no Fu* was present at the restrictive temperature in hh<sup>ts</sup> discs, indicating that conversion of Fu to Fu* in P cells is both Ptc independent and Hh dependent (Figure 1E).
Figure 3. Fu* Is Essential for Hh Function in the A Compartment

Ectopic expression of ptc in the P compartment quantitatively depleted Fu* from third instar wing imaginal discs (C) and caused wings to develop with fused veins (D), differing from wild-type (A) and apparently phenocopying fu1 wings (B). (They were ~8% smaller than wild-type wings but were not severely mis-shapen.) Ectopic expression of hh in the region of the A/P border quantitatively converted Fu to Fu* (E) and affected vein patterns and shape of the anterior wing (F). These morphological effects were suppressed when hh was ectopically expressed in a similar manner in a fu1 mutant (G). Arrows indicate vein 3-4 fusions (B, D, and G) and expansion of the region between veins 3 and 4 (F).

veins 3 and 4 is controlled by Hh (Jiang and Struhl, 1995; Li et al., 1995; Mullor et al., 1997), this phenotype is consistent with the expected consequences of an over-abundance of Hh. Interestingly, we found that the A compartment phenotype associated with ectopic expression of Hh-N was suppressed in a fu1 mutant background (Figure 3G) and was similar to fu1 (Figure 3B). These results suggest that Fu* is essential for normal patterning in the A compartment cells near the compartment border and that the phenotype caused by ectopic Hh signaling in the A compartment is mediated through Fu*.

To characterize the role of Ptc in Hh signal transduction, we monitored the distribution of Ptc and Hh in wing discs. We previously showed that Hh has a diffuse distribution in P cells where it is synthesized but that it coalesces in punctate structures in A border cells (Tábata and Kornberg, 1994). Similar studies have shown that Ptc also has a punctate distribution in the A border cells and that these punctate structures are likely to be endocytic vesicles (Capdevila et al., 1994). As shown in Figure 4, discs stained with anti-Ptc and anti-Hh antibodies reveal that Hh and Ptc colocalize in these particles. Moreover, after ectopic expression of Ptc in P compartment cells, a punctate distribution of both Ptc and Hh was found wherever cells contain both proteins (Figures 4D-4F).

The proposed role of Ptc as a negative regulator that limits the anterior spread of Hh (Chen and Struhl, 1996) leads to the prediction that overexpression of Ptc might counteract the influence of Hh on Fu. To test this possibility, we used the GAL4-UAS system to produce Ptc in the P compartment. Western analysis revealed that ectopic Ptc blocks conversion of Fu to Fu* (Figure 3C). Interestingly, wings of these Fu*-depleted en-GAL4; UAS-ptc flies (Figure 3D) had fused veins 3 and 4 and were indistinguishable from wings of fu1 flies (Figure 3B).

Discussion

Hh Signal Transduction

We studied the distribution of Fu*, the phosphorylated Fu isoform that is made when the Hh pathway is activated. Consistent with expectations, Fu* was absent from hh and smo mutant embryos in which Hh signal transduction is blocked (Figures 1A and 1B), and it accumulated in mutant embryos lacking Ptc, a negative regulator of Hh signaling (Figure 1C). These studies confirm Fu* as an indicator of Hh signaling (Thérond et al., 1996). In addition, we showed that ectopic expression of ptc in discs resulted in a fu phenocopy (Figure 3D) and abolished Fu* from the disc (Figure 3C). This indicates that Fu* embodies the active form of Fu. However, identification of the cells in normal wing discs that make Fu* did not conform to expectations.

Both Fu and Fu* were present in the A cells that express high levels of ptc at the A/P compartment border (Figure 1D). In contrast, only Fu was detected in A cells located away from the compartment border near the
Figure 4. Distribution of Hh and Ptc Proteins in Wing Imaginal Discs

Confocal microscopy of wing discs dissected from third instar larvae that were either wild-type (A–C) or that carried a HS-ptc P element and had been heat shocked (D–F) prior to staining with anti-Hh and anti-Ptc antibodies. In these focal planes, the Hh and Ptc proteins are in multi-vesiculate bodies. In the wild-type disc, particulate Hh (A) and Ptc (B) are distributed in a narrow band of A cells next to the A/P border and most of the Hh colocalizes with Ptc (C). Hh is also present in a diffuse distribution throughout the P compartment (Tabata and Kornberg, 1994). Ectopic expression of ptc distributes Ptc in intracellular vesicles throughout both A and P compartments (E) and redistributes Hh in P cells into Ptc-containing intracellular vesicles (D and F).
transduction pathway is activated independently of Hh in ptc mutant embryos. This behavior contrasts with P disc cells, which are Hh dependent and Ptc independent.

Two issues that may be relevant to this apparent contradiction are the role of Ptc and the mechanisms involved in transporting Hh from producing to receiving cells. Hh is presumed to bind Ptc, although no binding studies with the Drosophila proteins have been described. In the work reported here, indirect evidence for a Hh-Ptc interaction is provided. Hh adopts a diffuse distribution in P cells and a particulate appearance in A cells (Tabata and Kornberg, 1994). We show that Ptc and Hh colocalize to these particles (Figure 4) and that ectopic expression of ptc in P cells blocks signaling (Figure 3D), and redistributes Hh into Ptc-containing particles (Figure 4D). We do not know whether the Hh protein in these punctate structures signals or has been sequestered for lysosomal degradation or whether these particles are heterogeneous and have different functions. Our finding that P cells with a diffuse distribution of Hh produce Fu* while P cells with a particulate distribution of Hh do not shows that these particles do not correlate with signaling.

Perhaps the role of Ptc is in part to titrate Hh activity by targeting Hh to an endocytic pathway. This proposal places Ptc in a class of proteins that downregulates the signal that induces its own expression. Others in this class include Dad, an antagonist of Drosophila Dpp (Tsunzeizumi et al., 1997), Sprouty, an antagonist of Drosophila FGF (Hacohen et al., 1998), Argos, an antagonist of Drosophila EGF (Golembi et al., 1996), and Naked, an antagonist of Wg (Zeng et al., 2000). This model also suggests the presence of a Hh receptor other than Ptc that mediates signal transduction. The contrasting behavior of embryos and discs may reflect the use of different receptors, different regulatory components in the pathway, or the existence of compensating signaling systems in embryos that are not present in discs. Given the multiplicity of Hh binding proteins (Marigo et al., 1996; Stone et al., 1996; Chuang and McMahon, 1999) and the large and diverse group of organs in which Hh plays an instructive role, there may be significant heterogeneity in its downstream effectors.

Hh signaling in embryos and discs may also differ in the way they transport Hh to the target cells. The distances between Hh-producing cells and Hh-receiving cells does not exceed 2–3 cells in embryos, but may be significantly greater in discs. Different mechanisms may be used to move Hh over long distances or short, requiring distinct ways to engage the receptor. Further studies on the mechanisms that transport and bind Hh should resolve these issues.

Experimental Procedures

Western analysis was as described (Théron et al., 1996). Antibodies were rabbit anti-Fu (Robbins et al., 1997); rabbit anti-Ptc (R.L.J. Johnson and M.P. Scott); mouse anti-Ptc (Capdevila et al., 1994); rat anti-Cos2 (Sisson et al., 1997); goat anti-anti-rat HRP (Jackson Laboratories). Imaginal disc histology was performed as described (Tabata, 1994).

The following alleles were used in assays of mutant embryos, larvae and adults. hhnull is a deletion of most of the hh gene protein coding region (Lee et al., 1992). ptc1 carries a deletion of the ptc gene and refers to Df(2R)44CE. ptcA and ptcE refer to ptcnull and ptcnull, respectively, fuA (class I) allele; hh; hhnull allele (Ma et al., 1993); smo- embryos, smo502 (Alcedo et al., 1996). Genotypes of the GFP-balancers were: CKG19, CyO, P[FRT-40A]Kr-Gal4; P[FRT-40A]UAS-GFP.S65T] TKG4, TM3, Sb1; P[FRT-40A]Kr-Gal4; P[FRT-40A]UAS-GFP.S65T](Casso et al., 1999). Embryos were isolated as described (Casso et al., 1999). Parental genotypes: hh/hhTKG4, for hh; Df(2R)44CE, a; P[en-Gal4]; ptc/CG19, for Ptc; P[FRT-40A]hs-Flp[en-Gal4]; P[FRT-40A]neoFRT40A females and /Y; P[FRT-40A]neoFRT40A/CKG19 for smo; and hhnull/TM6, Tb for hhnull/hhnull; Df(2R)44CE/CG19; hhnull/TKG4 for hhnull; ptcnull/CG19; hhnull/TKG4 for hhnull; ptcnull/CG19; hhnull/TKG4 for hhnull.

To isolate A and P disc fragments, wing imaginal discs were isolated from wandering third instar larvae carrying en-Gal4 and UAS-GFP. Fluorescent fragments (P) and nonfluorescent fragments (A) were dissected with forceps. Purity of the fragments was confirmed by probing extracts with an anti-Cos2 antibody; the hyperphosphorylated isoform of Cos2 was detected only in the P fragments (data not shown), consistent with previous observations (Sisson et al., 1997). Experiments were repeated 12 times with equivalent results. Disc cells were isolated from third instar yw; ptcGal4/UAS-GFP larval wing discs and sorted by flow cytometry with methods similar to those of Ameiren and Axel (1997). Experimental results were confirmed in triplicate.

Preparation of double stranded RNAi and injection into embryos was carried out as described (Kennedale and Carthew, 1998). In all cases, at least 30 cuticles were analyzed, of which 60%–80% showed a cuticular conversion in response to the RNAi. The exceptions to this were ptc- embryos, which showed no response to hh RNAi.

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