Molecular Cell, Vol. 6, 479–485, August, 2000, Copyright ©2000 by Cell Press

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

F.-A. Ramírez-Weber,† D. J. Casso,†
P. Aza-Blanc, T. Tabata,‡ and T. B. Kornberg*
Department of Biochemistry and Biophysics
University of California
San Francisco, California 94143

Summary

***Drosophila* Hedgehog (Hh) is secreted by Posterior (P) compartment cells and induces Anterior (A) cells to create 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, 1998; 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

* To whom correspondence should be addressed (e-mail: tkornberg@biochem.ucsf.edu).
† These authors contributed equally to this work.
‡ Present address: Institute of Molecular and Cellular Biosciences, University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113-0032, Japan.

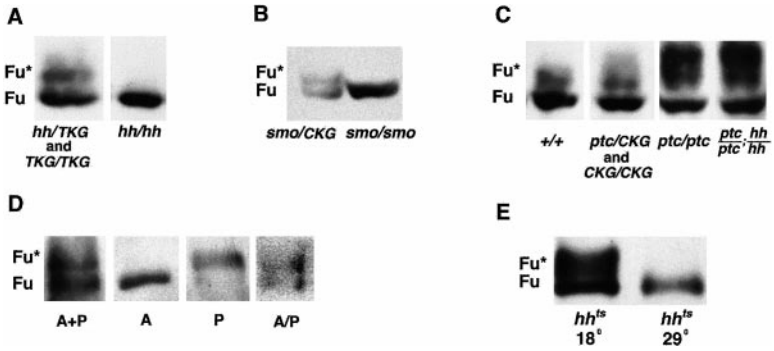

Figure 1. Production of Fu* Requires Hh and Smo but Not Ptc

Western blots were made using anti-Fu antibody and extracts from embryos (A-C) or wing imaginal discs (D and E). Fu* was quantitatively depleted in *hh* (A) and *smo* (B) embryos as well as in hh^{TS2} wing discs (E) that had been incubated at 29°C for 36 hr. Fu* was increased in *ptc* and *ptc;hh* backgrounds (C). The apparent difference between +/+ and *ptc*/balancer was not reproducible. Two forms of Fu* were resolved in these Western blots, the slowest mobility form increasing most in mutant extracts. The significance of the asymmetry of this change is not known. Fu* was present in P cells ([D], lane 3) and in A cells at the A/P border ([D], lane 4) but was absent from A cells not in proximity to the A/P border ([D], lane 2). Mutant *hh* and *ptc* embryos were identified by their lack of GFP fluorescence among the progeny of mutant/GFP- balancer parents; their GFP-containing siblings are heterozygous or homozygous for the GFP-balancer chromosomes. *smo*/+ females with germline clones produced nonfluorescent *(smo/smo)* and fluorescent *(smo/+)* progeny after mating with *smo*/GFP-balancer males. Alleles: +/+ (wild-type, Oregon R); hh^{ts2} (Ma et al., 1993); hh^{AC}, a null allele (Lee et al., 1992); *smo*, (smo^{Q14}); *ptc;* $(ptc^{CE}$; a deletion of *ptc*); hh^{ts}, (hh^{ts2}). Lanes contain the extracts of ~20 stage 9 embryos (A–C), 15 imaginal discs ([D], lane 1, and [E]), 20 dissected A or P fragments ([D], lanes 2 and 3), or 8×10^4 cells that contain GFP expressed under *ptc* control ([D], lane 4).

fluorescence. *hh* embryos selected by this method did not contain appreciable levels of Fu*, indicating that conversion of Fu to Fu* requires Hh (Figure 1A). This result confirms our previous observations with extracts of *hh* embryos that had been selected for their abnormal morphology at later embryonic stages (Thérond et al., 1996).

We also used the GFP-tagged balancer chromosomes to assess the roles of the Smo and Ptc proteins. Since eggs contain functionally significant levels of Smo (Alcedo et al., 1996; van den Heuvel and Ingham, 1996), *smo* germline clones were generated in *smo*/+ females to remove this maternal component, and matings were made to *smo*/GFP-balancer males. Fu, but no Fu*, was detected in extracts from homozygous *smo* mutant embryos, supporting the conclusion that Smo is essential for Hh signal transduction (Figure 1B). In contrast, *ptc* mutant embryos, selected as the nonfluorescent progeny of *ptc*/GFP-balancer parents, contained high levels of both Fu and Fu* (Figure 1C). The levels of Fu* were consistently and significantly increased, indicating that conversion to Fu* is not dependent on Ptc. This observation also supports the proposal that Ptc attenuates the Hh signal by repressing Hh and Smo (reviewed in Ingham, 1998) and supports the use of Fu* as a quantitative measure of Hh signaling.

Fu* produced in *ptc* mutants 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: ptc^{B}, which is a protein null mutant and ptc^{CE}, which is a *ptc* gene deletion. The *hh* allele, hh^{AC}, 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.

The State of the Hedgehog Pathway in *ptc* Mutants

The model that the Hh signaling pathway is activated in the absence of Hh when Ptc is also absent explains the *ptc*-like phenotype of *ptc;hh* double mutants (Ingham et al., 1991; Bejsovec and Wieschaus, 1993; Hooper, 1994). 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, ptc^{CE} and ptc^{B}, 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; Gorfinkiel 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* RNAi 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 ptc^{CE}/ptc^{CE} 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.

A Border Cells and P Cells Respond to Hh in Imaginal Discs

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

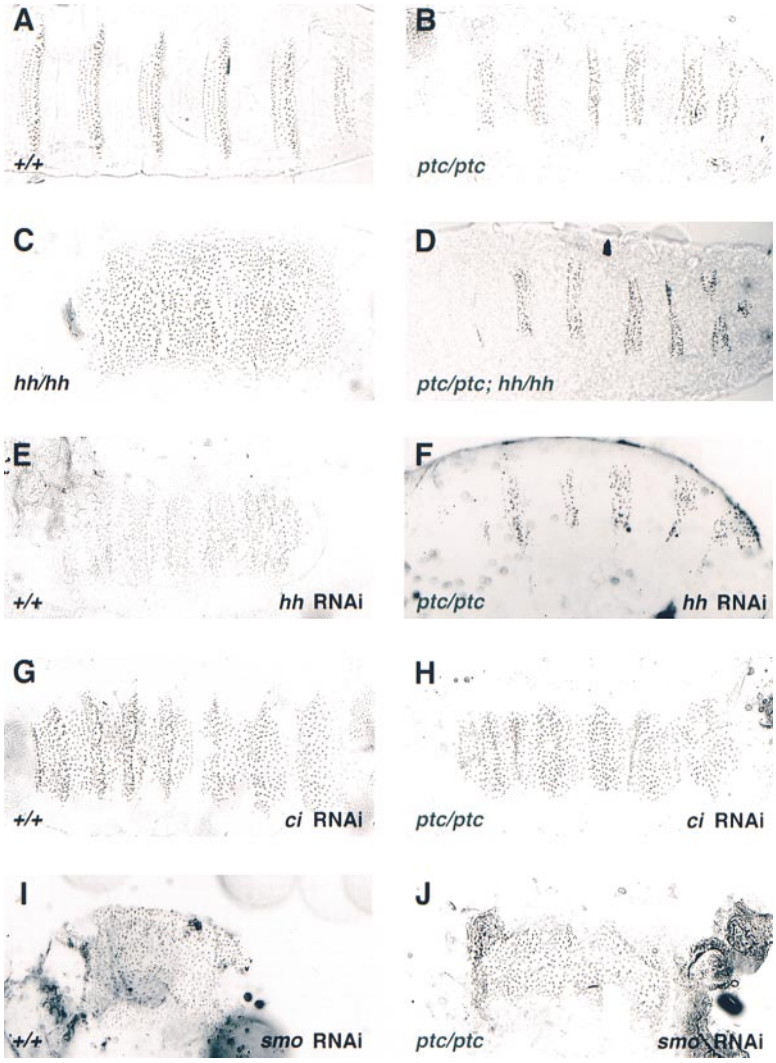

Figure 2. Embryonic Cuticles

Anterior is left in all panels. Genotypes are as follows: (A) wild-type; (B) ptc^{CE}/ptc^{CE}; (C) hh^{AC}/hh^{AC}; and (D) ptc^{CE}/ptc^{CE}; hh^{AC}/hh^{AC}. Note the similarity between the *ptc* and *ptc/hh* mutants. Similar results were obtained with ptc^{B}/ptc^{B}; hh^{AC}/hh^{AC} embryos, but ptc^{l}/ptc^{l}; hh^{AC}/hh^{AC} embryos had cuticle patterns that had characteristics of both *ptc* and *hh* (data not shown). These results are consistent with previous reports (Ingham et al., 1991; Bejsovec and Wieschaus, 1993; Hooper, 1994), and may suggest that ptc^{l} is not a null. RNAi was injected into embryos shown in (E)–(J). (E) and (F) show cuticles of wild-type (E) and ptc^{CE}/ptc^{CE} (F) embryos treated with *hh* RNAi; (G and H) of wild-type (G) and ptc^{CE}/ptc^{CE} embryos (H) treated with *ci* RNAi; and (I-J) of wild-type (I) and ptc^{CE}/ptc^{CE} embryos treated with *smo* RNAi.

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 Kornberg, 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^{ts} 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^{ts} discs, indicating that conversion of Fu to Fu* in P cells is both Ptc independent and Hh dependent (Figure 1E).

Ectopic Expresson of Ptc and Hh Modulates Fu Phosphorylation

To determine whether all of the Fu-containing cells in wing discs are responsive to Hh, wing discs were analyzed that expressed a diffusible form of Hh (Hh-N) (Jiang and Struhl, 1995; Li et al., 1995; Mullor et al., 1997) in the P compartment. Western analysis revealed that these discs contain Fu*, but no detectable Fu (Figure 3E). Wings produced by these discs had normal P compartments, but the A compartments had disrupted veins, abnormal folds, and excessive growth between veins 3 and 4 (Figure 3F). Since patterning of the region between

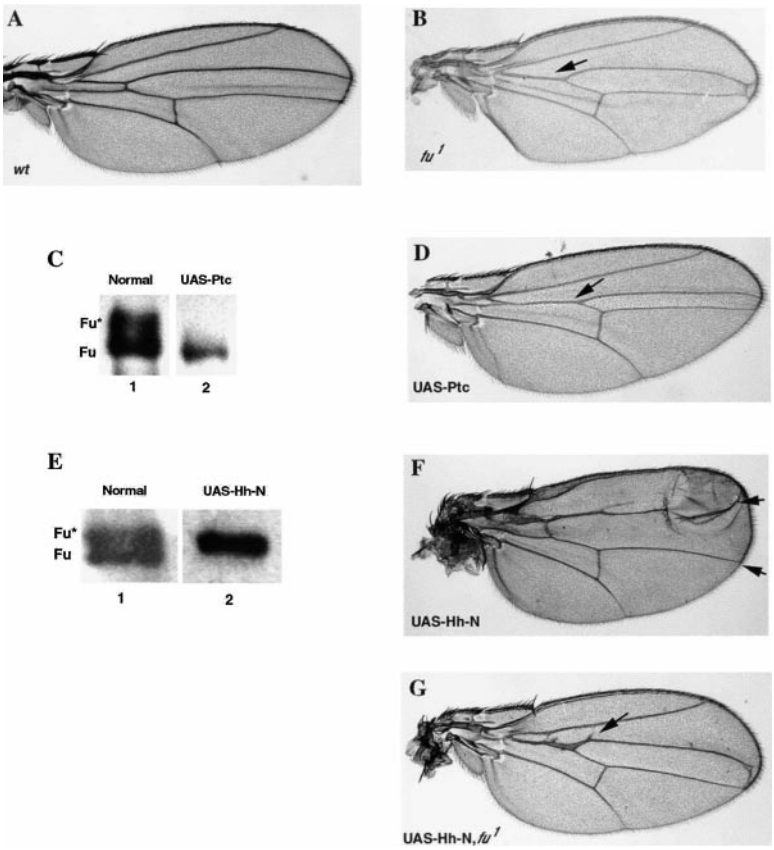

Figure 3. Fu* Is Essential for Hh Function in the A Compartment

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 fu^1 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 fu^1 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 overabundance of Hh. Interestingly, we found that the A compartment phenotype associated with ectopic expression of Hh-N was suppressed in a fu^1 mutant background (Figure 3G) and was similar to fu^1 (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 (Tabata 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 fu^1 flies (Figure 3B). Similar phenotypes are produced by flies expressing UAS-*ptc* with a *ptc*-GAL4 driver (data not shown; Johnson et al., 1995). These results suggest that Fu* is the functionally active isoform in the wing and that its production is tempered by Ptc.

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

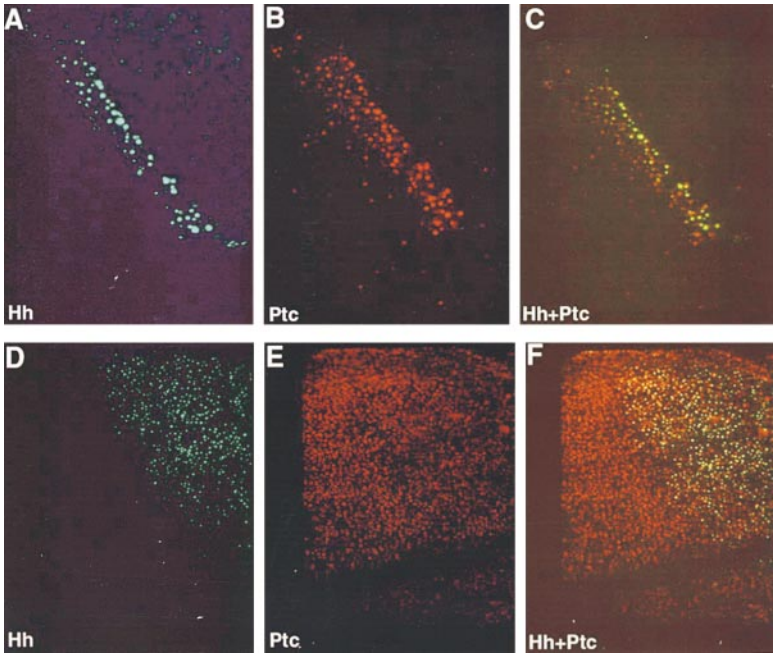

Figure 4. Distribution of Hh and Ptc Proteins in Wing Imaginal Discs

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).

disc flank, and only Fu* was detected in P cells. The quantitative conversion of Fu to Fu* in P cells shows that all of the Fu protein is responsive to Hh and indicates that P cells transduce the Hh signal. This latter conclusion contradicts a fundamental tenet of Hh signaling—that the cells that produce Hh do not transduce the Hh signal. P cells do not express Hh target genes such as *ptc* and *dpp*, so it had been assumed that they are refractory to Hh (Zecca et al., 1995). If the Hh signal transduction pathway is indeed active in P cells, as the presence of Fu* suggests, then the output of the pathway must be blocked at some downstream step. This is an unorthodox means of regulating a signal transduction pathway.

Although the Hh pathway is active in P cells, fu function is not required for normal development of the P compartment (Alves et al., 1998), and Hh signaling has no apparent role. We propose that the Hh signaling pathway does not reach transcriptional fruition in P cells due to the activity of Engrailed (En). En is expressed in P cells and induces these cells to express *hh*. P cells, as well as their neighbors in the A compartment, respond to Hh, initiate the Hh signal transduction cascade, and generate Fu*. In A border cells, Hh signal transduction modulates Ci to upregulate *dpp* and *ptc* expression. In contrast, En represses *ci* expression in P cells, thereby preventing a transcriptional response.

Several related observations support this model of Hh signaling in P cells. First, when En is absent from P cells, *ci*, *dpp*, and *ptc* are activated (Tabata et al., 1995). Presumably, En directly represses *ci* in normal P cells, and the expression of *ci* in the mutant cells mediates the induction of *dpp* and *ptc* as an indirect consequence of Hh signaling. It is also possible that En plays a direct role in repressing *dpp* and *ptc*, but the patterns in which *dpp* and *ptc* are induced at the periphery of *en* mutant clones suggests that their expression is dependent upon Hh (Tabata and Kornberg, 1994). Second, Hh seems to influence the activity of Ci when *ci* is expressed ectopically in P cells. Hh regulates Ci activity in part by converting Ci to an activator form (Ci^{Act}) and by inhibiting its conversion to a repressor form (Ci^{Rep}). When the full-length Ci protein was made ectopically in P cells, *dpp* and *ptc* were activated (Aza-Blanc et al., 1997) in a *smo*-dependent manner (Methot and Basler, 1999), and *hh*, a target of Ci^{Rep}, was not repressed. These observations indicate that Ci^{Act} is functional in these cells and that Ci^{Rep} is not. Both are hallmarks of Hh signaling. Using a temperature-sensitive allele of *hh*, our data with Fu* show that the state of the Hh signaling pathway is not constitutively activated in P cells, but that it reflects the activity of Hh.

Hh Signaling and the Role of Ptc

Ptc protein and *ptc* RNA have been detected only in A cells (Hooper and Scott, 1989; Nakano et al., 1989), so a role for Ptc in suppressing activation of the Hh pathway in the P cells of imaginal discs seems unlikely. For technical reasons, we have not been able to test this directly by examining Fu* in *ptc*- P disc cells, so we cannot rule out the possibility that P cells express *ptc* RNA and protein at levels that could not be detected. However, since the level of Ptc in P cells is much less than Smo, any model in which Ptc suppresses Smo signaling in the absence of Hh would require that Ptc act catalytically to silence Smo. If Ptc does act catalytically, it is not obvious why the much higher levels of Ptc in the A cells at the border fail to prevent Hh signaling. Moreover, the fact that overexpression of *ptc* depresses Hh signaling (Figures 4E and 4F) suggests that the relative levels of Hh and Ptc are important and directly influence Hh signaling. It therefore seems more likely that Hh signaling in discs is mediated by a Hh binding protein other than Ptc.

Previous work has shown that in embryos the Hh signal transduction pathway becomes Hh independent in the absence of Ptc (reviewed in Ingham, 1998). We examined several different *ptc;hh* allele combinations, made RNAi phenocopies of *hh* and *ci* in *ptc* mutants, and independently monitored Fu*. In each assay, the results were consistent with the proposal that Hh signal

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), suppresses the production of Fu* (Figure 3C), 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 (Tsuneizumi et al., 1997), Sprouty, an antagonist of *Drosophila* FGF (Hacohen et al., 1998), Argos, an antagonist of Drosophla EGF (Golembo 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 (Thérond et al., 1996). Antibodies were rabbit anti-Fu (Robbins et al., 1997); rabbit anti-Ptc (R.L. Johnson and M.P. Scott); mouse anti-Ptc (Capdevila et al., 1994); rat anti-Cos2 (Sisson et al., 1997); goat anti-rabbit HRP, and goat anti-rat HRP (Jackson Laboratories). Imaginal disc histology was preformed as described (Tabata, 1994).

The following alleles were used in assays of mutant embryos, larvae and adults. hh^{AC} is a deletion of most of the *hh* gene protein coding region (Lee et al., 1992). ptc^{CE} carries a deletion of the *ptc* gene and refers to *Df(2R)44CE*. ptc^{B} and ptc^{l} refer to ptc^{B98} and ptc^{IN108}, respectively. fu^{1}: fu^{62} (class I) allele; hh^{TS}:hh^{TS2} allele (Ma et al., 1993); *smo*- embryos, smo^{Q14} (Alcedo et al., 1996). Genotypes of the GFP-balancers were: *CKG19, CyO, P{w^{+mC}=Kr-Gal4}, P{w^{+mC}=UAS-GFP.S65T}; TKG4, TM3, Sb^{1}, P{w^{+mC}=Kr-Gal4}, P{w^{+mC}=UAS-GFP.S65T}* (Casso et al., 1999). Embryos were isolated as described (Casso et al., 1999). Parental genotypes: hh^{AC}/*TKG4*, for *hh*; Df(2R)44CE, al^{l}, dp^{ov1}, b^{1}, pr^{1}/*CKG19*, for *ptc*; *P{ry$^{+t7.2}$=hsFLP}12(l)/+; smo^{Q14}, P{ry$^{+t7.2}$=neoFRT}40A/smo^{+}, P{w^{+mC}=ovoD1–18}13X13a, P{w^{+mC}=ovoD1–18}13X13b, P{ry$^{+t7.2}$=neoFRT}40A* females and *+/Y; smo^{Q14}, P{ry$^{+t7.2}$=neoFRT}40A/CKG19* for *smo*; and hh^{TS2}/*TM6, Tb* for hh^{TS2}/hh^{TS2}; *Df(2R)44CE/CKG19;* hh^{AC}/*TKG4* for hh^{AC}, ptc^{CE}; ptc^{B98}/*CKG19;* hh^{AC}/*TKG4* for hh^{AC}, ptc^{B}. ptc^{IN108}/*CKG19;* hh^{AC}/*TKG4* for hh^{AC}, ptc^{l}.

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 Amerein and Axel (1997). Experimental results were confirmed in triplicate.

Preparation of double stranded RNAi and injection into embryos was carried out as described (Kennerdell 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.

Acknowledgments

We gratefully thank P. Dazon, HHMI FACS facility at UCSF; K.S. Ho and M.P. Scott for *UAS-Ptc* flies and anti-Cos2 antibody; K. Nybakken for anti-Fu antibody; R.L. Johnson, M.P. Scott, and I. Guerrero for anti-Ptc antibodies; J. Hooper for smo^{Q14}, FRT-40A flies; H.-Y. Lin and J. Gu for assistance with embryo injections; and the Bloomington Fly Stock Center. We also thank the following individuals for helpful discussions: K. Hill, S. Younger, G. Ehrenkaufer, B. Ng, F. Cifuentes, M. Singer, D. Robbins, and F. Chanut. This work was supported by grants to F.-A. R.-W. from UC Presidents Fellowship and to T. B. K. from the National Institutes of Health and Merck Genome Research Institute.

Received April 18, 2000; revised July 17, 2000.

References

Alcedo, J., Ayzenzon, M., Von Ohlen, T., Noll, M., and Hooper, J.E. (1996). The Drosophila smoothened gene encodes a seven-pass membrane protein, a putative receptor for the hedgehog signal. Cell *86*, 221–232.

Alves, G., Limbourg-Bouchon, B., Tricoire, H., Brissard-Zahraoui, J., Lamour-Isnard, C., and Busson, D. (1998). Modulation of Hedgehog target gene expression by the Fused serine-threonine kinase in wing imaginal discs. Mech. Dev. *78*, 17–31.

Aza-Blanc, P., Ramirez-Weber, F.A., Laget, M.P., Schwartz, C., and Kornberg, T.B. (1997). Proteolysis that is inhibited by hedgehog targets Cubitus interruptus protein to the nucleus and converts it to a repressor. Cell *89*, 1043–1053.

Basler, K., and Struhl, G. (1994). Compartment boundaries and the control of Drosophila limb pattern by hedgehog protein. Nature *368*, 208–214.

Bejsovec, A., and Wieschaus, E. (1993). Segment polarity gene interactions modulate epidermal patterning in Drosophila embryos. Development *119*, 501–517.

Bhat, K.M., and Schedl, P. (1997). Requirement for engrailed and invected genes reveals novel regulatory interactions between engrailed/invected, patched, gooseberry and wingless during Drosophila neurogenesis. Development *124*, 1675–1688.

Capdevila, J., Pariente, F., Sampedro, J., Alonso, J.L., and Guerrero, I. (1994). Subcellular localization of the segment polarity protein patched suggests an interaction with the wingless reception complex in Drosophila embryos. Development *120*, 987–998.

Casso, D., Ramirez-Weber, F.A., and Kornberg, T.B. (1999). GFP-tagged balancer chromosomes for Drosophila melanogaster. Mech. Dev. *88*, 229–232.

Chen, Y., and Struhl, G. (1996). Dual roles for patched in sequestering and transducing Hedgehog. Cell *87*, 553–563.

Chen, E.H., and Baker, B.S. (1997). Compartmental organization of the Drosophila genital imaginal discs. Development *124*, 205–218.

Chen, Y., Cardinaux, J.R., Goodman, R.H., and Smolik, S.M. (1999). Mutants of cubitus interruptus that are independent of PKA regulation are independent of hedgehog signaling. Development *126*, 3607–3616.

Chuang, P.T., and McMahon, A.P. (1999). Vertebrate Hedgehog signalling modulated by induction of a Hedgehog-binding protein. Nature *397*, 617–621.

Forbes, A.J., Lin, H., Ingham, P.W., and Spradling, A.C. (1996). hedgehog is required for the proliferation and specification of ovarian somatic cells prior to egg chamber formation in Drosophila. Development *122*, 1125–1135.

Golembo, M., Schweitzer, R., Freeman, M., and Shilo, B.Z. (1996). Argos transcription is induced by the Drosophila EGF receptor pathway to form an inhibitory feedback loop. Development *122*, 223–230.

Gorfinkiel, N., Sanchez, L., and Guerrero, I. (1999). Drosophila terminalia as an appendage-like structure. Mech. Dev. *86*, 113–123.

Hacohen, N., Kramer, S., Sutherland, D., Hiromi, Y., and Krasnow, M.A. (1998). sprouty encodes a novel antagonist of FGF signaling that patterns apical branching of the Drosophila airways. Cell *92*, 253–263.

Hooper, J.E. (1994). Distinct pathways for autocrine and paracrine Wingless signalling in Drosophila embryos. Nature *372*, 461–464.

Hooper, J.E., and Scott, M.P. (1989). The Drosophila *patched* gene encodes a putative membrane protein required for segmental patterning. Cell *59*, 751–765.

Ingham, P.W. (1998). Transducing Hedgehog: the story so far. EMBO J. *17*, 3505–3511.

Ingham, P.W., Taylor, A.M., and Nakano, Y. (1991). Role of the Drosophila *patched* gene in positional signalling. Nature *353*, 184–187.

Jiang, J., and Struhl, G. (1995). Protein kinase A and hedgehog signaling in Drosophila limb development. Cell *80*, 563–572.

Johnson, R.L., Grenier, J.K., and Scott, M.P. (1995). patched overexpression alters wing disc size and pattern: transcriptional and post-transcriptional effects on hedgehog targets. Development *121*, 4161–4170.

Kennerdell, J.R., and Carthew, R.W. (1998). Use of dsRNA-mediated genetic interference to demonstrate that frizzled and frizzled 2 act in the wingless pathway. Cell *95*, 1017–1026.

Lee, J.J., von Kessler, D.P., Parks, S., and Beachy, P.A. (1992). Secretion and localized transcription suggest a role in positional signaling for products of the segmentation gene hedgehog. Cell *71*, 33–50.

Li, W., Ohlmeyer, J.T., Lane, M.E., and Kalderon, D. (1995). Function of protein kinase A in hedgehog signal transduction and Drosophila imaginal disc development. Cell *80*, 553–562.

Ma, C., Zhou, Y., Beachy, P.A., and Moses, K. (1993). The segment polarity gene hedgehog is required for progression of the morphogenetic furrow in the developing Drosophila eye. Cell *75*, 927–938.

Marigo, V., Davey, R.A., Zuo, Y., Cunningham, J.M., and Tabin, C.J. (1996). Biochemical evidence that patched is the Hedgehog receptor. Nature *384*, 176–179.

Méthot, N., and Basler, K. (1999). Hedgehog controls limb development by regulating the activities of distinct transcriptional activator and repressor forms of Cubitus interruptus. Cell *96*, 819–831.

Mullor, J.L., Calleja, M., Capdevila, J., and Guerrero, I. (1997). Hedgehog activity, independent of decapentaplegic, participates in wing disc patterning. Development *124*, 1227–1237.

Nakano, Y., Guerrero, I., Hidalgo, A., Taylor, A., Whittle, J.R.S., and Ingham, P.W. (1989). A protein with several possible membrane-spanning domains encoded by the *Drosophila* segment polarity gene *patched*. Nature *341*, 508–513.

Ohlmeyer, J.T., and Kalderon, D. (1998). Hedgehog stimulates maturation of Cubitus interruptus into a labile transcriptional activator. Nature *396*, 749–753.

Price, M.A., and Kalderon, D. (1999). Proteolysis of cubitus interruptus in Drosophila requires phosphorylation by protein kinase A. Development *126*, 4331–4339.

Robbins, D.J., Nybakken, K.E., Kobayashi, R., Sisson, J.C., Bishop, J.M., and Therond, P.P. (1997). Hedgehog elicits signal transduction by means of a large complex containing the kinesin-related protein costal2. Cell *90*, 225–234.

Sisson, J.C., Ho, K.S., Suyama, K., and Scott, M.P. (1997). Costal2, a novel kinesin-related protein in the Hedgehog/Patched signaling pathway. Cell *90*, 235–245.

Stone, D.M., Hynes, M., Armanini, M., Swanson, T.A., Gu, Q., Johnson, R.L., Scott, M.P., Pennica, D., Goddard, A., Phillips, H., et al. (1996). The tumour-suppressor gene patched encodes a candidate receptor for Sonic hedgehog. Nature *384*, 129–134.

Suzuki, T., and Saigo, K. (2000). Transcriptional regulation of *atonal* required for *Drosophila* larval eye development by concerted action of Eyes absent, Sine oculis and Hedgehog signaling independent of Fused kinase and Cubitus interruptus. Development *127*, 1531–1540.

Tabata, T., and Kornberg, T.B. (1994). Hedgehog is a signaling protein with a key role in patterning Drosophila imaginal discs. Cell *76*, 89–102.

Tabata, T., Schwartz, C., Gustavson, E., Ali, Z., and Kornberg, T.B. (1995). Creating a Drosophila wing de novo, the role of engrailed, and the compartment border hypothesis. Development *121*, 3359–3369.

Thérond, P.P., Knight, J.D., Kornberg, T.B., and Bishop, J.M. (1996). Phosphorylation of the fused protein in response to signaling from hedgehog. Proc. Natl. Acad. Sci. *93*, 4224–4228.

Tsuneizumi, K., Nakayama, T., Kamoshida, Y., Kornberg, T.B., Christian, J.L., and Tabata, T. (1997). Daughters against dpp modulates dpp organizing activity in Drosophila wing development. Nature *389*, 627–631.

van den Heuvel, M., and Ingham, P.W. (1996). smoothened encodes a receptor-like serpentine protein required for hedgehog signalling. Nature *382*, 547–551.

Zecca, M., Basler, K., and Struhl, G. (1995). Sequential organizing activities of engrailed, hedgehog and decapentaplegic in the Drosophila wing. Development *121*, 2265–2278.

Zeng, W., Wharton, K.A., Jr., Mack, J.A., Wang, K., Gadbaw, M., Suyama, K., Klein, P.S., and Scott, M.P. (2000). *naked cuticle* encodes an inducible antagonist of Wnt signalling. Nature *403*, 789–795.