Genetic Dissection of the Drosophila Cubitus interruptus Signaling Complex

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Much of our understanding of the Hedgehog (Hh) signaling pathway comes from Drosophila, where a gradient of Hh signaling regulates the function of the transcription factor Cubitus interruptus (Ci) at three levels: protein stabilization, nuclear import, and activation. Regulation of Ci occurs in a cytoplasmic complex containing Ci, the kinesin-like protein Costal-2 (Cos2), the serine-threonine kinase Fused (Fu), and the Suppressor of Fused [Su(fu)] protein. The mechanisms by which this complex responds to different levels of Hh signaling and establishes distinct domains of gene expression are not fully understood. By sequentially mutating components from the Ci signaling complex, their roles in each aspect of Ci regulation can be analyzed. The Cos2-Ci core complex is able to mediate Hh-regulated activation of Ci but is insufficient to regulate nuclear import and cleavage. Addition of Su(fu) to the core complex blocks nuclear import while the addition of Fu restores Hh regulation of Ci nuclear import and proteolytic cleavage. Fu participates in two partially redundant pathways to regulate Ci nuclear import; the kinase function plays a positive role by inhibiting Su(fu), and the regulatory domain plays a negative role in conjunction with Cos2.

Key Words: Hedgehog; Ci; Fu; Su(fu); signal transduction; nuclear import; pattern formation.

INTRODUCTION

The Hedgehog (Hh) signal transduction pathway was first defined by its role in patterning the embryonic segments of Drosophila (Ingham et al., 1991). Subsequent work has shown that it plays a central role in many developmental processes including appendage formation (Ruiz i Altaba, 1999). In Drosophila, appendages are derived from imaginal discs which are divided into two compartments, anterior and posterior (Garcia-Bellido et al., 1973; Crick and Lawrence, 1975). Cells in the posterior compartment express the secreted protein Hh but are not themselves Hh responsive (Dominguez et al., 1996). All anterior compartment cells are Hh responsive (Basler and Struhl, 1994), and those near the compartment boundary, which receive Hh, activate a signal transduction cascade that ultimately leads to changes in gene transcription.

Signaling is initiated at the plasma membrane where Hh binds its receptor Patched (Ptc) (Chen and Struhl, 1999) and relieves Ptc repression of the serpentine protein Smoothened (Smo). At modest levels of Hh signaling, the Cubitus interruptus (Ci) protein is stabilized and not cleaved into a repressor form (Aza-Blanc et al., 1997). In this report, Ci will refer to the full-length Ci155 transcriptional activator. Cleavage requires phosphorylation of Ci by protein kinase A (PKA) (Chen et al., 1998; Price and Kalderon, 1999), and the presence of the kinesin-like protein, Costal-2 (Cos2) (Wang and Holmgren, 1999; Methot and Basler, 2000), and the F-box/WD40 repeat protein Slimb (Jiang and Struhl, 1998). A modest level of Hh signaling also leads to the release of Ci from the cytoplasm and its rapid shuttling into and out of the nucleus (Methot and Basler, 2000; Wang and Holmgren, 2000; Wang et al., 2000). Cos2 is necessary for the sequestration of Ci in the cytoplasm (Chen et al., 1999; Wang and Holmgren, 2000; Wang et al., 2000), and the release of Ci from this sequestration is dependent on the ser/thr kinase encoded by fused (fu) (Methot and Basler, 2000; Wang and Holmgren, 2000; Wang et al., 2000).

While full-length Ci is a transcriptional activator, high levels of Hh signaling cause the Ci protein to become a more potent transcription factor (Ohlmeyer and Kalderon, 2001).
We call this form activated Ci (Wang and Holmgren, 1999). The process of activation requires phosphorylation of Ci by PKA and the presence of Cos2 (Wang and Holmgren, 2000). Activation of Ci by Hh signaling has been shown to be independent of inhibition of Ci cleavage (Methot and Basler, 1999) and promotion of Ci nuclear trafficking (Wang and Holmgren, 2000). This complex pattern of regulation results in the formation of at least two distinct domains of gene expression (Hepker et al., 1997; Vervoort et al., 1999; Wang and Holmgren, 1999). Immediately adjacent to the compartment boundary, activated Ci directs high-level ptc expression, activation of collier (Vervoort et al., 1999), late expression of en (Blair, 1992), and activation of an artificial Ci reporter construct 4bs-lacZ (Hepker et al., 1999). In a broader domain, stabilization and cytoplasmic release of Ci lead to the expression of decapentaplegic (dpp). Away from Hh signaling, Ci is cleaved into a repressor form, which prevents expression of dpp away from the compartment boundary (Methot and Basler, 1999).

Cos2 is at the center of Ci regulation. Biochemical studies have shown that it forms a complex with Ci, Fu, and Suppressor of fused [Su(fu)], and that Hh signaling leads to the release of this complex from microtubules (Robbins et al., 1997; Sisson et al., 1997; Monnier et al., 1998; Stegman et al., 2000). As with Ptc and Smo, Cos2 appears to be involved in all aspects of Hh regulation of Ci (Wang and Holmgren, 1999; Wang and Holmgren, 2000). Su(fu) was identified by its ability to suppress the wing vein defect of fu class I alleles (mutations in the kinase domain) (Preat, 1992). Su(fu) has almost no phenotype on its own but in combination with fu class II alleles (mutations in the regulatory domain) causes a phenotype similar to cos2 (Preat et al., 1993). Several vertebrate homologs of Su(fu) have been identified and shown to impede the nuclear localization of Gli1 (Ding et al., 1999; Kogerman et al., 1999; Stone et al., 1999; Murone et al., 2000). In Drosophila, it has also been proposed that Su(fu) and Cos2 play redundant roles in sequestering Ci in the cytoplasm (Stegman et al., 2000), and recent studies have shown that Su(fu) protein can block entry of Ci into the nucleus and that the kinase function of Fu opposes this function (Methot and Basler, 2000).

Here, we examine how Cos2, Fu, and Su(fu) interact and regulate Ci activity. Our results suggest that Fu plays two opposite roles in the regulation of Ci release from its cytoplasmic tether. In addition to inhibiting Su(fu) via its kinase function, Fu exerts a second, probably structural function, via its regulatory domain which is also required for the regulation of Ci proteolysis. Although Fu is an important link between Hh signaling and Ci regulation, we further demonstrate that it is not the only link. By simultaneously mutating Fu and Su(fu), we show that the Cos2-Ci core complex is able to mediate Hh activation of Ci. Taken together, our results suggest that while the components of the Ci signaling complex have intercalating roles, their functions are separable. Distinct components appear to be required for sensing different levels of Hh signaling and eliciting different responses.

MATERIALS AND METHODS

Stocks

\( fu^{m4} \) was isolated by RAH in an EMS screen. Su(fu)\(^{b} \) behaves as a genetic null and deletes the region encoding the C-terminal domain of the protein (Preat, 1992). The mutation appears to be a protein null as no protein is detected with a polyclonal antibody (Stegman et al., 2000). The Su(fu)\(^{b} \) line was obtained from the Bloomington Stock Center. The \( fu^{m63} ; fu^{m63};FRT40A fu^{m4} \), and \( fu^{m63};FRT40A fu^{m4} \) lines were obtained from D. Kalderon.

Western Blotting

\( y w^{u4} \) and \( y w^{u4};Su(fu)^{b} \) mutant larvae were identified by the color of their mouth hooks. C1\(_{10} \) and C1\(_{2} \), were detected with 1C\(_{10} \) (1:10), a monoclonal anti-Ci antibody (Wang and Holmgren, 1999). All secondary antibodies were purchased from The Jackson Laboratory.

Clonal Analysis

The genotypes of the larvae used in clonal analysis are as follows:

- \( fu^{m4};FRT18A/myc \); hsflp\(^{+} \) (on 3)
- \( fu^{m4}; smo; fu^{m4}/Y ; FRT40A myc/FRT40A smo \)
- \( fu^{m63};DC0^{f2}; y w hsflp fu^{m63}/Y ; FRT40A y^{+} fu^{m4}/FRT40A DC0^{f2} myc \)
- \( fu^{m63}; cos2^{2}; y w hsflp fu^{m63}/Y; FRT42D y^{+} fu^{m4}/FRT42D cos2^{1} myc \)

LMB treatment was performed as previously described (Wang and Holmgren, 2000). Ci antibody stainings of imaginal discs were performed with the monoclonal antibody 2A1 (1:1) (Motzny and Holmgren, 1995) which only recognizes the full-length form of Ci.

Analysis of Hh Target Genes

decapentaplegic (dpp) expression was followed by using a lacZ enhancer trap and a rabbit anti-\( \beta \)-galactosidase antibody (1:2000) (Buenzow and Holmgren, 1995), ptc expression was followed by using a monoclonal antibody to the Ptc protein (1:1000) (gift from I. Guerrero), and 4bs-lacZ was followed by using a rabbit anti-\( \beta \)-galactosidase antibody.

Images were collected on a Zeiss Axiophot fluorescence microscope and deconvolved using VayTek software.

RESULTS

The \( fu \) gene has been extensively characterized (Preat et al., 1993; Therond et al., 1996). Class I alleles are suppressed by Su(fu) and map to the putative kinase domain (amino acids 1-268) while class II alleles, in combination with Su(fu), have a cos2-like phenotype. Class II alleles map either to the regulatory domain (amino acids 269-805) or cause deletion of both kinase and regulatory domain se-
sequences. In earlier work, we have used the fu94 mutation because it gives a particularly strong phenotype. Molecular characterization of this allele showed that it is a 5-bp deletion at amino acid position 351 which removes almost the entire regulatory domain.

fu Is Required for Ci Cleavage in the Absence of Hh Signaling

All fu alleles have been associated with elevation of Ci protein levels, though different alleles vary in how far into the anterior compartment the elevation is observed. In imaginal discs mutant for fu94, Ci protein levels are elevated across the entire anterior compartment (see Fig. 2B). A similar phenotype has been observed in fu6, another class II allele (Alves et al., 1998). In fuRX15, also a class II allele, Ci is elevated in a broad domain, but not in the most anterior region of the disc (Wang and Holmgren, 1999). In contrast, although class I alleles (kinase domain mutants) such as fuRX15 elevate the level of Ci above that of the wild-type Ci stripe, the elevation is observed only along the compartment boundary and does not cause an expansion of the Ci stripe (Ohlmeyer and Kalderon, 1998; Alves et al., 1998). The high uniform levels of Ci in fu94 mutants result from a marked increase in the levels of full-length Ci (Fig. 1). It has been shown with fuRX15 that the elevated Ci levels are dependent upon Hh signaling and decrease in clones that are also mutant for smo (Ohlmeyer and Kalderon, 1998). This is not the case for fu94, where Ci levels remain high in cells far away from Hh signaling as well as in the absence of smo activity (Figs. 2A–2C). Since ptc expression is significantly compromised in fu94 mutant discs (see Fig. 4D) and Ptc is known to limit its range of Hh action (Chen and Struhl, 1998), it is possible that Hh signaling extends much further into the anterior compartment in these mutants. By examining fu94 mutant clones away from the compartment boundary in otherwise wild-type discs, the range of Hh signaling should be restricted to its normal range, and the effect of fu94 on Ci protein levels can be assayed in the absence of Hh. An example of such a mutant clone (Figs. 2D–2F) shows very high levels of Ci protein. Therefore, the Fu regulatory domain is required for the processing of Ci in the absence of Hh signal. Some Ci is still cleaved into the Ci75 repressor form (Fig. 1), and this appears sufficient to prevent ectopic expression of dpp (data not shown) and is consistent with the lack of disc overgrowth.

Previous studies have shown that fu is required for the release of Ci from a cytoplasmic tether (Methot and Basler, 2000; Wang and Holmgren, 2000; Wang et al., 2000). If release is a prerequisite for cleavage, the inhibition of Ci proteolytic processing in fu94 mutants could be due to the lack of release and not to a role of fu in Ci processing per se. To distinguish between these hypotheses, fu94;Su(fu)LP double mutants were generated. As described below, in these double mutants, Ci is released and can translocate into the nucleus. Therefore, if release is a prerequisite for cleavage, these discs would provide a context in which Ci is available for proteolysis even in the absence of fu. Figure 1 shows that there is a dramatic increase in the level of full-length Ci relative to Ci75 in fu94, while in Su(fu)LP the levels of both full-length Ci and Ci75 are decreased. In fu94;Su(fu)LP double mutants, there is a reduction in Ci protein levels relative to fu94, but processing of full-length Ci to Ci75 is still inhibited. These results suggest that in addition to regulating Ci release, fu appears to be directly required for the efficient processing of Ci into Ci75. In its absence, some Ci75 is generated, but the great majority of Ci remains full length.

Su(fu) and fu Do Not Act in a Simple Linear Pathway

Nuclear import of Ci was assayed in discs treated with leptomycin B (LMB) (Kudo et al., 1998) which blocks Ci nuclear export. In anterior cells along the compartment boundary, Hh signaling allows Ci to shuttle into and out of the nucleus. After treatment with LMB, these cells show nuclear accumulation of Ci (Fig. 3A). In contrast, Ci is still sequestered in the cytoplasm of cells away from the boundary (Methot and Basler, 2000; Wang and Holmgren, 2000; Wang et al., 2000). In fu class I (Fig. 3B) and class II mutant discs (fuRX15, Fig. 3C) (fu94, Wang and Holmgren, 2000), Ci remains tethered in the cytoplasm throughout the anterior
compartment and fails to accumulate in the nucleus after LMB treatment. Discs mutant for Su(fu)

\( \text{Su(fu)}_{\text{LP}} \) and treated with LMB appear wild type, with Ci nuclear along the compartment boundary and cytoplasmic away from Hh signaling (Fig. 3D). In \( \text{fu}^{94} \) (a class I allele);\( \text{Su(fu)}_{\text{LP}} \) double mutants treated with LMB, Ci was cytoplasmic away from Hh signaling and was distributed uniformly across the nucleus and cytoplasm in cells adjacent to the compartment boundary (Fig. 3E). We interpret the rather uniform subcellular distribution of Ci along the compartment boundary as a partial suppression of the \( \text{fu}^{94} \) nuclear import defect by \( \text{Su(fu)}_{\text{LP}} \). This would explain the ability of \( \text{Su(fu)}_{\text{LP}} \) to rescue the wing vein defect of class I \( \text{fu} \) mutants and is consistent with the hypothesis that the kinase domain of \( \text{Fu} \) antagonizes the function of \( \text{Su(fu)} \), a negative regulator of Ci release. In combination with \( \text{Su(fu)}_{\text{LP}} \), class II \( \text{fu} \) mutants show ectopic expression of dpp and have a wing disc overgrowth phenotype (Alves et al., 1998). In the case of \( \text{fu}^{94} \);\( \text{Su(fu)}_{\text{LP}} \), this phenotype is particularly severe with ectopic expression of dpp throughout the anterior compartment (Figs. 4A, 4E, and 4F). While the decrease in the levels of Ci could account for the ectopic expression of dpp (Methot and Basler, 1999), we also wanted to examine the subcellular distribution of full-length Ci. In \( \text{fu}^{94} \);\( \text{Su(fu)}_{\text{LP}} \)

**FIG. 2.** Stabilization of Ci is Hh-independent in \( \text{fu}^{94} \) mutants. In all, anterior is to the left and dorsal is up. (A–C) A \( \text{fu}^{94} \) mutant wing imaginal disk in which smo mutant clones have been generated (arrows), smo clones are marked by the loss of Myc staining (A), Ci protein is visualized using the monoclonal antibody 2A1, which only recognizes the full-length form (B). Ci levels are elevated across the entire anterior compartment and are not lowered in smo mutant clones, which can no longer transduce the Hh signal. (The typical stripe of elevated Ci along the compartment boundary can be seen in E.) (C) A merge of (A) and (B). (D–F) A \( \text{fu}^{94} \) mutant clone away from the compartment boundary. The mutant clone is marked by the loss of Myc staining (D) and Ci protein is visualized in (E). Ci protein levels are elevated in the absence of Hh signal. (F) A merge of (D) and (E).
double mutant discs treated with LMB, Ci protein was nuclear throughout the anterior compartment (Fig. 3F). Thus, removal of the Fu regulatory domain and Su(fu) from the signaling complex causes Ci to be released independent of Hh signal. This synthetic phenotype differs from the phenotype of either single mutant, suggesting that the functions of Fu and Su(fu) in the complex cannot be fully explained by a linear epistatic relationship in which the Fu kinase function regulates Su(fu). Imaginal discs double mutant for fu<sup>Rx15</sup>;Su(fu)<sup>LP</sup> have a very similar phenotype (Fig. 3G). An interesting aspect of this result is that while fu<sup>Rx15</sup> mutants only have a broad stripe of elevated Ci levels, the double mutants have uniform high-level Ci throughout the anterior compartment.

Su(fu) Requires Cos2 to Sequester Ci in the Cytoplasm

To examine the relationship between Su(fu) and Cos2 in the sequestration of Ci in the cytoplasm, clones double mutant for fu<sup>mH63</sup>, a class I allele, and cos2<sup>1</sup> (also known as cos2<sup>W1</sup>) were generated. In the fu<sup>mH63</sup> background, the Fu kinase function should be absent, and Su(fu) would be expected to constitutively hold Ci in the cytoplasm (Methot and Basler, 2000). By also removing cos2, it is possible to determine whether Su(fu) is sufficient for Ci retention. Discs containing fu<sup>mH63</sup>;cos2<sup>1</sup> double mutant clones were subjected to LMB, and as with cos2<sup>1</sup> single mutants (Wang and Holmgren, 2000), Ci accumulates in the nucleus of double mutant clones independent of Hh signaling (Fig. 3H). The fu<sup>mH63</sup>;cos2<sup>1</sup> double mutant cells have quite high levels of Ci protein, and we have previously shown that it is possible to saturate the Ci binding sites in the cytoplasm (Wang and Holmgren, 2000). Therefore, as a control to show that the nuclear Ci is not just a result of having very high Ci protein levels, fu<sup>mH63</sup>;DCO<sup>H2</sup> mutant clones were generated. These clones have even higher levels of Ci yet it remains cytoplasmic (Fig. 3I). These results support a growing body of evidence that Cos2 is critically required for sequestering Ci in the cytoplasm (Robbins et al., 1997; Sisson et al., 1997; Monnier et al., 1998; Chen et al., 1999; Stegman et al., 2000).

Hh Regulation of Ci Activation Still Occurs in fu<sup>94</sup>;Su(fu)<sup>LP</sup> Double Mutants

The expression of ptc (Fig. 4B) and that of 4bs-lacZ (Fig. 4C), a reporter construct (Hepker et al., 1999), can be used to assay Ci activation (Methot and Basler, 1999; Wang and Holmgren, 1999). In Su(fu)<sup>LP</sup> mutants, the stripe of high-level ptc expression is still present directly adjacent to the compartment boundary (data not shown). 4bs-lacZ is also expressed normally along the compartment boundary of the wing pouch (data not shown). In fu<sup>94</sup>, ptc expression is reduced (Fig. 4D) and 4bs-lacZ expression is completely eliminated (data not shown). However, it had not been possible to determine whether Ci activation is affected in fu mutants, since the defects in Ci nuclear import may be sufficient to cause these phenotypes. It appears that in fu<sup>94</sup>, cells next to the compartment boundary still respond to Hh signaling in some way, since there remains a broad stripe of elevated ptc expression along the A/P boundary (Fig. 4D). fu<sup>94</sup>;Su(fu)<sup>LP</sup> double mutants provide an opportunity to study the role of fu in Ci activation, as in these mutants, Ci protein is released and enters the nucleus, making it possible to separate the effect of blocking nuclear import from that of blocking activation. High-level ptc expression is observed along the compartment boundary in fu<sup>94</sup>;Su(fu)<sup>LP</sup> mutants (Fig. 4G). This is in contrast to a broad low-level stripe of ptc expression that is observed in fu<sup>94</sup> single mutants (Fig. 4D). The expression of 4bs-lacZ is weak but consistent in the fu<sup>94</sup>;Su(fu)<sup>LP</sup> double mutants (Fig. 4H). The weakness of 4bs-lacZ expression may be a consequence of the lower levels of total Ci protein in the Su(fu)<sup>LP</sup> background. Therefore, activation of Ci by the Hh signal transduction cascade can occur independent of Fu and Su(fu) regulation of proteolysis and nuclear import. The opposite result is observed in cos2<sup>1</sup> mutants in which Ci is released but cannot be activated by Hh signaling (Wang and Holmgren, 2000). Thus, it appears that the Ci protein can interact with Cos2 in the absence of Fu and Su(fu) function and be activated in response to Hh signaling.

DISCUSSION

Fu, Su(fu), Cos2, and Ci form a cytoplasmic signaling complex that is crucial for the integration of different aspects of Ci regulation. In this paper, we examined class I and class II alleles of fu for their interaction with Su(fu) and cos2. From these experiments, we are able to model how each of the components in the complex functions to mediate Hh signal transduction. The Ci-Cos2 Core Complex Is Sufficient for Hh Regulation of Ci Activation

In fu<sup>94</sup>;Su(fu)<sup>LP</sup> mutants, it is unlikely that either Fu or Su(fu) is present in the complex, as Robbins et al. (1997) have shown that Fu protein from class II mutants fails to immunoprecipitate Cos2, and Segman et al. (2000) were unable to detect Su(fu) protein in Su(fu)<sup>LP</sup> mutants. In this mutant combination, the processing of Ci is not Hh regulated, and this results in uniform levels of Ci protein across the entire anterior compartment (see Fig. 4E). Hh regulation of Ci nuclear import is also lost, and the Ci protein shuttles into and out of the nucleus throughout the anterior compartment (Fig. 5A). As a consequence, dpp is expressed at modest levels in all anterior compartment cells. Previous studies have shown that Cos2 is required for Ci sequestration in the cytoplasm and its proteolytic processing, but clearly Cos2 is not sufficient for all aspects of Ci regulation. In the absence of the Fu regulatory domain and Su(fu) from the complex, all anterior compartment cells behave as if they are receiving at least modest levels of Hh signaling.
While dpp is expressed throughout the anterior compartment, there is still Hh regulation of transcriptional activation. The elevated expression of ptc and the activation of 4bs-lacZ in cells immediately adjacent to the compartment boundary are similar to the wild-type situation, and even dpp expression is higher in cells receiving Hh signaling. Thus, the process of Ci activation appears intact in the absence of Hh regulation of Ci cleavage or nuclear import. This observation contrasts with the disruption of ptc and 4bs-lacZ expression in cos2 clones, in which Ci also accumulates to high levels and enters the nucleus (Wang and Holmgren, 2000). The requirement of cos2 for ptc expression has subsequently been supported by others (Wang et al., 2000). Taken together, Cos2 is necessary and sufficient for Ci activation in response to high-level Hh signaling. It is likely that Cos2 provides a platform where Ci and other components required for Ci activation, such as PKA (Wang and Holmgren, 2000), can assemble.

**FIG. 3.** Regulation of Ci nuclear import by fu, cos2, and Su(fu). All wing discs in this figure were treated with LMB for 1–2 h and the subcellular distribution of Ci assayed with the 2A1 monoclonal antibody. In wild-type discs, Ci accumulates in nuclei in response to Hh signaling (A). In animals mutant for either fuH63 (a class I allele) or fuRX15 (a class II allele), Ci remains cytoplasmic throughout the anterior compartment (B and C, respectively). In Su(fu)RX15, the subcellular distribution of Ci is relatively normal, cytoplasmic away from Hh signaling and nuclear along the compartment boundary (D). In fuRX15;Su(fu)RX15 double mutants, Ci protein is cytoplasmic away from Hh signaling and has a uniform pattern along the compartment boundary (E). On the other hand, in fuH63;Su(fu)RX15 mutants, Ci accumulates in the nuclei throughout the anterior compartment (F). In fuH63;Su(fu)RX15 mutant discs, Ci is also nuclear throughout the anterior compartment (G). In fuH63;cos21 double mutant clones, Ci protein is always nuclear (H). Shown are two clones away from the compartment boundary. To exclude the possibility that such Hh-independent nuclear import is simply due to the highly elevated Ci protein levels in these clones, clones double mutant for fuH63;DC0 (DC0 encodes the catalytic subunit of PKA) were generated as controls. PKA does not have a role in Ci nuclear import but is required for Ci proteolytic cleavage. In fuH63;DC0 double mutant clones, Ci is also elevated to very high levels but remains cytoplasmic (I).
Su(fu)-Ci-Cos2 Triplex Tethers Ci in the Cytoplasm and Is Unresponsive to Hh Signaling

Addition of Su(fu) to the Ci-Cos2 complex dramatically reduces the rate of Ci release from the complex as Ci does not accumulate in the nucleus in fu94 mutant discs that have been treated with LMB. No regulation of Ci nuclear import is observed, and processing of Ci into Ci75 is still inhibited (Fig. 5B). The block in Ci nuclear import by Su(fu) appears to be dependent on the presence of Cos2 as clones double mutant for fu94;cos2 release Ci independent of Hh signaling.

Fu-Ci-Cos2 Triplex Is Able to Mediate All Aspects of Ci Regulation

Addition of Fu to the Ci-Cos2 complex essentially restores Hh regulation of Ci nuclear import and the processing of Ci into Ci75. Therefore, in the absence of Hh signaling, Fu is required for both the cleavage of Ci into Ci75 and its retention in the cytoplasm (Fig. 5C). The major consequence of removing Su(fu) from the complex is a significant decrease in the overall levels of both Ci and Ci75. This decrease does not appear to significantly compromise Hh regulation.

The Regulatory Domain of Fu Assists Cos2 in the Cytoplasmic Tethering of Ci

Although Cos2 provides an important tethering force, it apparently cannot hold Ci in the cytoplasm on its own. As discussed above, addition of either the Fu regulatory domain or Su(fu) is sufficient to restore effective tethering. The requirement for Fu in Ci tethering is a new finding, as it has been shown that Fu plays a positive role in Ci nuclear entry by inhibiting Su(fu) via its kinase domain. Further

**FIG. 4.** fu and Su(fu) are not required for Ci activation. The wild-type wing disc patterns of dpp-lacZ (anti-β-galactosidase), Ptc (anti-Ptc), and 4bs-lacZ (anti-β-galactosidase) are shown in (A), (B), and (C), respectively. In fu94 mutants, ptc is expressed in a broad weak stripe along the compartment boundary of the wing disc (anti-Ptc; D). The weak activation of ptc suggests that the small amount of Ci that is released in fu94 can be activated by Hh signaling. In fu94;Su(fu) double mutants, wing imaginal discs show an overgrowth phenotype and have high levels of Ci throughout the anterior compartment (visualized with the monoclonal antibody 2A1 in E). In these double mutants, dpp is expressed throughout the anterior compartment (anti-β-galactosidase; F), while high-level ptc expression is only observed directly adjacent to the compartment boundary (anti-Ptc; G). The double mutants also have low-level expression of the Ci reporter 4bs-lacZ along the compartment boundary (anti-β-galactosidase; H).
examination of different classes of fu alleles demonstrates that Fu participates in Ci tethering through its regulatory domain. When a Fu class I mutant protein (kinase domain mutations) is added to the Ci-Cos2 core complex [fu\textsuperscript{94}, Su(fu)\textsuperscript{LP}], regulation of Ci nuclear entry is almost wild type (Fig. 3E). In contrast, when a Fu class II mutant protein (regulatory domain mutations) is present [fu\textsuperscript{RX15}, Su(fu)\textsuperscript{LP}], the complex fails to tether Ci in the absence of Hh signaling (Figs. 3F and 3G). It has been previously shown that Fu interacts with Cos2 through its regulatory domain (Robbins et al., 1997), and the proteins made by fu class II alleles fail to immunoprecipitate with Cos2. These results suggest that the interaction between Cos2 and the Fu regulatory domain is important for Cos2 to tether Ci in the absence of Su(fu) activity. This Cos2-Fu interaction may also be important for targeting Fu kinase regulation of Su(fu). Both fu\textsuperscript{RX15} and fu\textsuperscript{94}, which delete different extents of the regulatory domain (Preat et al., 1993), might be expected to retain kinase function, yet Hh regulation of Su(fu) appears to have been lost and Ci is not released from the cytoplasm in either of these mutants. The simplest explanation is that by preventing Fu interaction with Cos2, Fu cannot perform its structural role in the complex nor can it regulate Su(fu). Thus, Fu plays two opposite roles in the regulation of Ci nuclear entry. Without Hh signaling, the regulatory domain in conjunction with Cos2 tethers Ci in

FIG. 5. Model for the regulation of Ci release. Events that are regulated by high-level Hh signaling are shown in red while responses to modest levels of Hh signaling are shown in blue. (A) In the absence of the Fu regulatory domain and Su(fu), the Core complex of Ci and Cos2 is still able to respond to high-level Hh signaling and activate Ci. This depends on the presence of PKA which is also required for Ci cleavage. We venture to speculate that Cos2 plays a key role in bringing Ci and PKA together. The rapid nuclear import of Ci and the severe block in its proteolytic cleavage, both of which normally depend upon modest levels of Hh signaling, occur constitutively in this background. We do not know whether the N-terminal kinase domain still present in fu\textsuperscript{94} mutants can associate with the complex, though results from Robbins et al. (1997) suggest that this might not be the case. (B) Addition of Su(fu) to the complex dramatically reduces the release of Ci from the complex, but does not significantly alter the proteolytic processing or activation of Ci. (C) Addition of Fu to Cos2 and Ci restores Hh regulation of Ci nuclear import and cleavage. Fu kinase function is not required for this activity. Whether addition of Fu modifies the response of Cos2 to Hh signaling or establishes a new pathway is an open question. (D) In wild-type cells, an additional mode of Hh regulation is added which regulates Ci nuclear import and requires the Fu kinase function and Su(fu).
the cytoplasm; upon Hh signaling, the kinase domain inhibits Su(fu) which, along with a change in the Cos2/Fu regulatory domain interaction, leads to the release of Ci.

**Requirement for Fu in the Processing of Ci into the Ci75 Repressor**

In addition to its role in regulating Ci release, Fu also has a role in regulating Ci proteolysis. This is not dependent on the Fu kinase domain, as Ohlmeyer and Kalderon (1998) showed in the fu class I mutation, fu<sup>6146</sup>, that Ci is readily cleaved in the absence of Hh signaling. Methot and Basler (2000) implicated the C-terminal regulatory domain in this process by showing that a fu class II mutation, fu<sup>6</sup>, blocks repression of hh expression in the absence of Hh signaling. With the fu<sup>64</sup> mutation, all anterior compartment cells fail to efficiently process Ci. Using fu<sup>64/ Su(fu)<sup>19</sup>, we further showed that this proteolytic processing defect is separable from the Ci release defect also observed in fu mutants. As with nuclear import, the structural role of Fu in Ci processing most likely involves interaction with Cos2.

Taking the nuclear import and proteolytic processing results together, it appears that the Fu protein is required for the complex to behave properly in the absence of Hh signaling (Fig. 5). Elimination of the Fu regulatory domain leads to a block in Ci processing, and in combination with elimination of Su(fu), release of Ci. These are events which normally require modest levels of Hh signaling.

**Additional Roles of Su(fu) in the Regulation of Ci**

While it has been possible to clearly establish a role for Su(fu) in Ci nuclear import, its role in Ci activation and cleavage is less clear. In cells double mutant for cos2; Su(fu), Ci appears to be at least partially activated as double mutant clones away from the compartment boundary ectopically express en (Wang et al., 2000). A reasonable interpretation of these data is that Ci activation is inhibited by Su(fu) and signaling through Cos2 relieves such inhibition. But this cannot be the whole story. In Su(fu)<sup>19</sup> discs, the expression of ptc or en is still tightly regulated and does not expand into all the cells with efficient Ci nuclear import. This regulation of Ci activity is evidently not rendered by the Fu regulatory domain, as it persists in the fu<sup>64</sup>;Su(fu)<sup>19</sup> double mutants (Figs. 4G and 4H). It seems likely that Su(fu) is partially redundant with other factors that regulate Ci activation and that these yet to be identified factors function with Cos2 in the fu;Su(fu) double mutants. Su(fu) may also play some role in Ci cleavage. In the fu<sup>64</sup>;Su(fu)<sup>19</sup> double mutants, the level of Ci seems significantly reduced relative to fu<sup>64</sup> single mutants. In addition, Ci protein levels are not elevated across the entire anterior compartment in fu<sup>6145</sup> single mutants but are in fu<sup>6145</sup>;Su(fu)<sup>19</sup> double mutants. The implication of Su(fu) in these other aspects of Hh regulation suggests that while it is possible to dissect the complex and assign primary roles to the various components, the complex does normally function as a whole.

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