INTRODUCTION

Members of the Hedgehog (Hh) family of secreted signaling proteins function as potent organizers in animal development. Drosophila Hh controls pattern formation during segmentation and limb development. Vertebrate Hh homologues play key roles in the morphogenesis of the neural tube, somites, axial skeleton, limbs, lung and skin (reviewed in Neumann and Cohen, 1997; Ingham, 1998; Ruiz i Altaba, 1999; McMahon, 2000). Most if not all Hh signaling is mediated through the transcriptional effectors of the Gli/Ci family (reviewed by Aza-Blanc and Kornberg, 1999). Major interest is devoted therefore to the mechanisms by which Hh signaling regulates the activity of Gli/Ci proteins.

The function of Hh is best understood in the developing Drosophila wing imaginal disc, a tissue that is formed by two cell populations, the anterior (A) and posterior (P) compartments. The selector gene engrailed (en) is expressed in P cells where it permits the synthesis of Hh while at the same time preventing a response to Hh (reviewed by Neumann and Cohen, 1997; Ingham, 1998; Ruiz i Altaba, 1999; McMahon, 2000). Most if not all Hh signaling is mediated through the transcriptional effectors of the Gli/Ci family (reviewed by Aza-Blanc and Kornberg, 1999). Major interest is devoted therefore to the mechanisms by which Hh signaling regulates the activity of Gli/Ci proteins.

Hh diffuses into the A compartment and initiates a signaling cascade by binding to its receptor, the multi-transmembrane domain protein Patched (Ptc). Hh neutralizes the inhibitory hold of Ptc on the co-receptor protein Smoothened (Smo), which then signals to downstream effectors (reviewed in Alcedo and Noll, 1997; Ingham, 1998; Strigini and Cohen, 1999). Signaling results in the stabilization of the zinc finger protein Cubitus interruptus (Ci; Orenic et al., 1990) and the transcriptional activation of target genes. Several proteins, such as Cos2, PKA, Su(fu) and Slimb, prevent or downregulate signaling, but their mode of action is not well understood. Some evidence suggests that Cos2 tethers Ci to the cytoplasm (Robbins et al., 1997, Sisson et al., 1997), whereas Slimb and PKA are required for the proteolytic processing of Ci (Jiang and Struhl, 1998; Chen et al., 1998). Cleavage of Ci occurs in the absence of Hh (Aza-Blanc et al., 1997) and results in the release of a C-terminally truncated Ci (here referred to as Ci[rep]), that translocates to the nucleus and inhibits transcription of target genes such as decapentaplegic (dpp) and hh itself (Dominguez et al., 1996; Aza-Blanc et al., 1997; Méthot and Basler, 1999). Reception of Hh prevents Ci[rep] formation, and activates the transcription of several Hh target genes such as dpp and ptc (Aza-Blanc et al., 1997; Méthot and Basler, 1999). Recently, it has been shown that inhibition of Ci processing is not sufficient to activate Hh-target gene transcription. An uncleavable form of Ci (CiU) still requires Hh input to induce ptc expression in wing imaginal discs (Méthot and Basler, 1999), and the proteosome inhibitor lactacystin does not potentiate the response to Hh in tissue culture cells (Chen C.-H. et al., 1999). These results suggest that a Hh-dependent event other than prevention of cleavage permits full-length Ci to function as a potent transcriptional activator (Ci[act]). Several studies identified phosphorylation of Ci by protein kinase A (PKA) as a key event in mediating cleavage (Chen et al., 1998; Chen Y. et al., 1999; Price and Kalderon, 1999;
DNA was obtained by fusing EGFP DNA to the 5'S891, S892 and S962. The cDNA for CiGFP was made by fusing an following serine residues are replaced by alanines: S838, S855, S856, from C. Dahmann and encodes a mutant protein in which the

Our previous studies focussed on the demonstration that the formation of both Ci[act] and Ci[rep] is tightly controlled by Hh signaling (Méthot and Basler, 1999). Here we examined Hh signal transduction components for their role in the conversion of full-length Ci into either Ci[act] or Ci[rep]. We found that the formation of Ci[rep] requires PKA, Cos2 and Fu, while the generation of Ci[act] proceeds through the neutralization of PKA and Cos2 activity. Although Ci can bypass PKA and gain constitutive Ci[act] activity by mutations in its PKA phosphorylation sites, we have observed that this activity can be further stimulated by Hh signaling. Both Su(fu) and Fu alter the transcriptional output of this mutant form of Ci, by regulating its nuclear-cytoplasmic localization. The accumulation of full-length Ci in the nucleus is Hh-dependent and is blocked by excess Su(fu). We propose that Fu kinase stimulates the Hh pathway, not by promoting the formation of Ci[act], but rather by facilitating its entry into the nucleus.

MATERIALS AND METHODS

Transgenes
Ectopic expression of Ci, Ci mutants, mycSu(fu), mycFu and mycCos2 in wing imaginal discs was achieved with the UAS-Gal4 system (Brand and Perrimon, 1993). UAS-Ci and UAS-CiPKA4 (in this paper referred to as CiPKA4), where Ci codons for amino acids 837, 838 and 839 were mutated to encode alanine residues, have been described in Méthot and Basler (1999). The cDNA for CiPKA4 is a gift from C. Dahmann and encodes a mutant protein in which the following serine residues are replaced by alanines: S838, S855, S856, S891, S892 and S962. The cDNA for CiGFP was made by fusing an EGFP (Clontech) cDNA in frame to the 3’ end of Ci cDNA. GFP-Ci75 DNA was obtained by fusing EGFP DNA to the 5’ end of Ci cDNA that had been truncated at the unique EcoRV site. All constructs that were obtained following a PCR step or blunt ending of restriction sites were sequenced to verify integrity. DNA templates used for in vitro translations were obtained from Ci cDNA coding for a triple HA tag at its 5’ end (Méthot and Basler, 1999), and truncated at its unique EcoRV site, to generate pBS-Ci440. The in vitro translated product derived from this construct consists of a triple HA tag followed in frame by Ci amino acids 5 to 440. Deletions (A212-268 and A268-346) were generated by PCR mutagenesis and introduced into pBS-Ci440. Fused and Su(fu) cDNAs were amplified from Drosophila embryonic cDNA and cloned in frame 5’ to a myc tag. The resulting vectors pBS-mycFu and pBS-mycSu(fu) were transferred as Asp718-XbaI fragments into pUAST. A construct coding for GST-mycSu(fu) was derived from pBS-mycSu(fu), where an EcoRI-blunted Asp718 fragment was transferred into pGEX3X (Pharmacia) digested with BamHI, blunted and redigested with EcoRI. Cos2 cDNA (gift of K. Ho and M. Scott) was cloned in frame 3’ to a myc-tag, and transferred to pUAST.

Reporter genes used in this study were dppP10638 (Zecca et al., 1995), hhP110 (Lee et al., 1992), ptcP1085L3 (Chen and Struhl, 1996). Gal4 drivers used were C765 (Nellen et al., 1996), act5c>CD2>Gal4 (Pignoni and Zipursky, 1997) and apterous-Gal4 (Calleja et al., 1996).

Protein purification, GST pull-down assays
pGEX-mycSu(fu) was transformed into E. coli BL21. Bacteria were grown to an optical density of 0.5 to 0.8 before induction with 1 mM isopropyl-ß-D-galactopyranosid (IPTG). Cells were grown for 3 hours at 30°C before harvesting by centrifugation. The pellet was resuspended in lysis buffer (phosphate-buffered saline, 1 mM EDTA, 1 mM DTT and 1 mM phenylmethlysulfonyl fluoride). Cells were lysed on ice by five sonication cycles. Cellular debris was removed by centrifugation. GST-fusion proteins were purified on glutathione-agarose (Pharmacia). Ci deletion mutants (as specified in the figure legend) were labeled with [35S]methionine using the T7 TNT in vitro translation system (Promega). Translation reactions (20 µl) were diluted to 300 µl in binding buffer (20 mM Hepes pH 7.4, 200 mM KCl, 1 mM DTT, 1 mM MgCl2, 1% NP40, 10% glycerol) and incubated for 1 hour at room temperature with 1 µg of GST-fusion protein. Glutathione-agarose beads were added, and the mixture was incubated for an additional 15 minutes on a rotator. Beads were washed five times in binding buffer, before loading on an SDS-polyacrylamide gel. Gels were fixed, treated for fluorography and exposed overnight on Kodak XAR-5 film.

Marked clones of mutant cells and immunohistochemistry
Alleles used in this study were: smo1, smo2 (Nüsslein-Volhard et al., 1984); DCOE95 (Lane and Kalderon, 1993); slimb1 (Jiang and Struhl, 1998); cos25 (Forbes et al., 1993); fu1, fu6, fu1663 and Su(fu)LP (Préat et al., 1993). Clones of mutant cells were generated by Flp-mediated mitotic recombination (Xu and Rubin, 1993). Larvae were heat shocked in late first instar for 30 minutes at 35°C and heat shocked again in late 3rd instar phase to induce marker gene expression (hs70-GFP or hs70-CDF2). Following a 1 hour recovery period, larvae were dissected, discs were fixed and stained with the appropriate antibodies. The following antibodies were used: rat monoclonal anti-Ci (gift from R. Holmgren); mouse monoclonal anti-Ptc (gift from I. Guerrero); mouse monoclonal and rabbit polyclonal anti-GFP (Clonetech); mouse monoclonal anti-Ci20X34 (Sorotech); mouse monoclonal (Promega) and rabbit polyclonal (Cappel) anti-ßGal; mouse monoclonal anti-myc 9E10; Alexa 488 and 594 secondary antibodies (Molecular Probes).

Presence of Ci[rep] was assayed genetically in vivo by its effect on hh-lacZ transcription. The final genotype of larvae were the following:

Fig. 1A: y w hs70-flp; smo3 FRT39/hsp70-CD2 FRT39; UAS-Ci/ C765 hh-lacZ
Fig. 1B: y w hs70-flp; smo3 DCOE95/hsp70-CD2 FRT39; UAS-Ci/ C765 hh-lacZ
Fig. 1C: y w hs70-flp; smo3 FRT42 cos25/smo3 FRT42 P[sma4, hsp70-GFP]; UAS-Ci/ C765 hh-lacZ
Fig. 1D: y w fia4; smo3 FRT39/hsp70-CD2 FRT39; UAS-Ci hh-lacZ/ C765 hs70-flp
Fig. 3C: y w hs70-flp; smo3 FRT39/hs70-CD2 FRT39; UAS-CiPKA4/ C765 hh-lacZ.

To determine the Hh-dependency of CiPKA4, larvae with the following genotype were analyzed:

Fig. 2A: DCOE95; y w hs70-flp; dpp-lacZ; FRT42 hs70-CD2/ FRT42 cos25
Fig. 2B: PKA5; y w hs70-flp; FRT40 DCOE95 / 2x hs70-mpyc
Fig. 2C: dpp-lacZ; y w hs70-flp; FRT40 DCOE95 / 2x hs70-mpyc

Clones expressing mycFu, mycSu(fu) and mycFuΔN were produced by mild heat shock (30 minutes at 34°C) of larvae containing the respective UAS construct and the act5c>CD2>Gal4 transgene (Pignoni and Zipursky, 1997). To inhibit nuclear export, discs from 3rd instar larvae were dissected and cultured for 8 hours in C8 media (Bhanot et al., 1996) in the presence or absence of 30 nM leptomycin B.
RESULTS

Formation of Ci[rep] requires Cos2, PKA and Fu

Ci functions as a potent repressor of hh expression in the absence of Hh signal transduction (Aza-Blanc et al., 1997; Méthot and Basler, 1999). Posterior cells that ectopically express Ci transduce the Hh signal and do not form Ci[rep] due to their exposure to the Hh signal. Removal of Smo function, however, prevents such P cells from transducing Hh and results in the formation of Ci[rep] and inhibition of hh transcription (Fig. 1A). To determine which components of the Hh signal transduction cascade are required for the generation of Ci[rep], we expressed Ci in P cells and removed the function of Smo together with that of either Cos2 or PKA. In contrast to smo- single mutant clones, hh transcription was not reduced in smo-PKA- (Fig. 1B) or smo-cos2- (Fig. 1C) double mutant clones.

![Fig. 1. Cos2, PKA and Fu are required to make Ci[rep].](Image)

Hence, the activities of PKA and Cos2 are required for Ci[rep] formation.

The behavior of certain fu alleles suggests that Fu, in addition to its stimulatory function, also plays an inhibitory role in the Hh pathway (Préat et al., 1993). Two types of fu alleles exist. Molecular analysis of these two classes has shown that fu type I alleles bear mutations in the kinase domain, which are thought to reduce or abolish kinase activity, while fu type II alleles lack the C terminus of the protein (Théront et al., 1996). We assayed Ci[rep] formation in posterior smo- cells that expressed Ci and were either mutant for a fu type I (fu1) or a type II (fu5) allele. hh transcription was eliminated in smo- clones of fu type I discs (data not shown), but not of fu type II discs (Fig. 1D). Thus, the catalytic function of Fu is dispensable for Ci[rep] formation, whereas the function of the C terminus is required for Ci[rep].

Formation of Ci[act] is prevented by PKA and Cos2

We found that absence of Cos2 or PKA activity prevents formation of Ci[rep]. It has been shown earlier that mere prevention of Ci cleavage does not suffice for Ci[act] formation (Méthot and Basler, 1999). To test whether, in these mutant contexts, prevention of Ci[rep] formation is linked to the generation of Ci[act], we removed PKA or Cos2 and assessed Ci[act] activity. While the repression of hh transcription is indicative for the presence of Ci[rep], the upregulation of ptc transcription serves as an indicator for the presence of Ci[act] (Méthot and Basler, 1999). We examined ptc expression in A cells mutant for cos2 or PKA and found, in both cases, ptc to be upregulated (Fig. 2B,C). These results indicate that A cells mutant for cos2 or PKA generate Ci[act], and suggest that neutralization of the activities or effects of either Cos2 or PKA is an important step for the formation of Ci[act]. Interestingly, although the C terminus of Fu is required for Ci[rep] formation, its absence did not lead to ectopic ptc-lacZ expression and Ci[act] formation (Fig. 2D).

Ci protein resistant to phosphorylation by PKA is still subject to Hh control

Ci[act] is generated in cells that lack PKA activity (Li et al., 1995; Jiang and Struhl, 1995; this work). An equivalent situation can be created by mutating the PKA phosphorylation sites of Ci. We mutated one (CiPKA1) or four (CiPKA4) PKA phosphorylation sites and compared the ability of these mutants to activate ptc-lacZ in wing imaginal discs. Ubiquitous weak expression of wild-type Ci led to ptc-lacZ activation only in Hh-exposed cells (Fig. 3A). This indicates that under physiological conditions, transcriptional activity of Ci is under the control of Hh. In contrast, CiPKA1 activated ptc-lacZ in all cells, regardless of their exposure to Hh (Fig. 3B). Thus, CiPKA1 is constitutively active in vivo. Identical results were recently described by several groups, and are taken as indication for a role of PKA in Ci activation (Chen Y. et al., 1999; Price and Kalderon, 1999, Wang et al., 1999). Interestingly, closer examination of discs ubiquitously expressing CiPKA1 reveals that P cells express ptc-lacZ at higher levels than A cells (Figs 3B, 4I). This suggests that the transcriptional activity of CiPKA1 can be further enhanced by Hh signaling. We have obtained similar results with CiPKA4 (Figs 3C, 4C). To test whether it is the reception of the Hh signal that stimulates the basal activity of CiPKA, we removed
the function of Smo from a subset of P cells expressing CiPKA4. Such cells transcribed ptc-lacZ at lower levels than their smo+ neighbors, levels that were similar to those found in anterior smo+ CiPKA cells (Fig. 3C). Thus Ci protein that is constitutively active due to mutated PKA phosphorylation sites is further stimulated in its transcriptional activity by the Hh signal.

**Fu kinase activity stimulates CiPKA**

It is possible that the reduction of ptc-lacZ expression in posterior smo− CiPKA4 cells is due to formation of some Ci[rep], which could compete with the Ci[act] activity of CiPKA. We used the repressor assay described above to test whether CiPKA4 can be converted to a transcriptional repressor, and found that hh transcription in smo− posterior cells was essentially unaffected (Fig. 3D). This indicates that CiPKA4 cannot be converted to Ci[rep] and that the reduction of ptc expression in smo− CiPKA4 cells is not due to the presence of Ci[rep].

Next, we searched for components of the Hh pathway that could stimulate the basal activity of CiPKA. Fu is one of the few proteins that have a positive input on Hh signaling (Préat et al., 1990, Ohlmeyer and Kalderon, 1998; Théron et al., 1999). We examined ptc-lacZ levels in wing imaginal discs that ubiquitously expressed CiPKA1, in either a wild-type or fu1 background. ptc-lacZ expression was clearly reduced in P cells of fu1 discs (compare Fig. 4A and B). Similar results were also obtained with CiPKA4 (Fig. 4I,J). Slightly elevated ptc-lacZ could still be seen near the AP compartment boundary in fu1 discs, and may be the result of cumulative activities of endogenous Ci[act] and CiPKA. We conclude that Fu kinase enhances the basal activity of CiPKA.

**Fu stimulates CiPKA by inhibiting Su(fu) activity**

fu is tightly linked to Su(fu), both genetically and biochemically (Préat, 1992; Préat et al., 1993; Monnier et al., 1998). To test whether the modulation of CiPKA activity...
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involves Su(fu), we ubiquitously expressed CiPKA4 together with myc-tagged Su(fu) or GFP as negative control. CiPKA4 (in the presence of GFP) induced ptc-lacZ expression everywhere in the wing imaginal disc but at higher levels in the P compartment (Fig. 4C). Co-expression with myc-Su(fu) abolished ptc-lacZ expression in the A compartment and reduced ptc-lacZ levels in P cells (Fig. 4D). Thus Su(fu) inhibits the activity of CiPKA4. This result is strengthened by the converse experiment, where the absence of Su(fu) (in Su(fu)P homozygous animals) reduced the difference in ptc-lacZ levels between A and P CiPKA1-expressing cells (Fig. 4E).

To determine whether Su(fu) negatively acts on CiPKA4 by direct protein-protein interaction, we created a mutant form of Ci with impaired Su(fu) binding. Su(fu) interacts with Ci within a region that encompasses amino acids 244-346 (Monnier et al., 1998). Indeed, an N-terminal deletion fragment (wild-type aa 5-440) or GST-Su(fu) (lanes 5, 7, 9), were pulled-down with either GST (lanes 4, 6, 8) or GST-Su(fu) (lanes 5, 7, 9). Subcellular localization of CiPKA4 (green) in wild-type salivary glands (G) or Su(fu)P mutant glands (H). Induction of ptcZ (red) by CiPKA4 marks nuclei. A marked increase in nuclear CiPKA4 is observed in salivary glands that lack Su(fu). The Hh-stimulated activity of CiPKA4 (I) is strongly reduced in fu mH63 mutant discs (J). The lower transcriptional activity of CiΔNPKA4 relative to CiPKA4 is probably due to lower expression levels of the protein. The Hh-stimulated activity of CiΔNPKA4 is reduced in fu mH63 discs (L), but much less so than that of CiPKA4 (compare J and L).

Fig. 4. Fu is needed to fully activate CiPKA. The Hh-dependent, high ptcZ levels (red) induced by CiPKA1 in P cells (A) are suppressed in discs mutant for fu (B). Stainings in A and B were done in parallel. The higher amounts of Ci protein (green) seen in B may be due to stabilization of Ci by Su(fu) (Ohlmeyer and Kalderon, 1998). (C,D) Co-expression of Su(fu) (D), but not GFP (C), reduced the ptcZ transcription induced by CiPKA4. (E) ptcZ expression in Su(fu) mutant discs expressing CiPKA1. Anterior and posterior ptcZ levels are similar. However, in most discs examined, P cells still expressed slightly more ptcZ. (F) GST pull-down assay with N-terminal deletion mutants of Ci and GST-Su(fu). Deletion of Ci amino acids 212-268 impaired the association between Ci and GST-Su(fu). In vitro translated Ci N-terminal fragments (wild-type aa 5-440 (lanes 1, 4, 5), or deleted between aa 212-268 (lanes 2, 6, 7) or 268-346 (lanes 3, 8, 9)), were pulled-down with either GST (lanes 4, 6, 8) or GST-Su(fu) (lanes 5, 7, 9). Subcellular localization of CiPKA4 (green) in wild-type salivary glands (G) or Su(fu)P mutant glands (H). Induction of ptcZ (red) by CiPKA4 marks nuclei. A marked increase in nuclear CiPKA4 is observed in salivary glands that lack Su(fu). The Hh-stimulated activity of CiPKA4 (I) is strongly reduced in fu mH63 mutant discs (J). The lower transcriptional activity of CiΔNPKA4 relative to CiPKA4 is probably due to lower expression levels of the protein. The Hh-stimulated activity of CiΔNPKA4 is reduced in fu mH63 discs (L), but much less so than that of CiPKA4 (compare J and L).
CiPKA4, to create CiΔNPKA4. This mutant was constitutively active, with P cells expressing higher ptc-lacZ levels than A cells (Fig. 4K). The activity of CiΔNPKA4 was slightly reduced when introduced into a strong fu background (fu^{mH63}; compare Fig. 4K and L), but the reduction was much less pronounced compared to that observed for CiPKA4 (compare Fig. 4I and J). We conclude that inhibition of Su(fu) activity by Fu kinase is an important step towards stimulating the basal activity of CiPKA4 (and by analogy Ci[act]).

**Ci, Fu and Cos2 shuttle in and out of the nucleus**

Regulation of Ci nuclear accumulation may be an important control point in the Hh signaling cascade. It has been shown recently that human GLI1 and Ci accumulate in the nuclei of tissue culture cells treated with the nuclear export inhibitor drug leptomycin B (LMB; Kogerman et al., 1999; Chen, C.-H. et al., 1999). To test if LMB has similar effects in vivo, Ci tagged at its C terminus with GFP (CiGFP) was expressed in the dorsal compartment of wing imaginal discs using an apterus-Gal4 driver (Calleja et al., 1996). Nuclear β-galactosidase derived from the ptc-lacZ reporter was used as a marker for nuclei and CiGFP activity. Under these conditions, CiGFP induced ptc-lacZ in P compartment cells only, indicating that the protein is active and under the control of Hh signaling (Fig. 5A, inset). A higher magnification shows that GFP fluorescence was largely excluded from the nuclei of untreated anterior cells, whereas some GFP fluorescence was observed in some nuclei of posterior cells (Fig. 5A, green). Treatment of wing discs with LMB resulted in a dramatic accumulation of GFP fluorescence in the nuclei of P cells or A cells close to the Hh source. A weaker accumulation can also be observed in A cells far from the Hh source (Fig. 5B). Several conclusions can be drawn from these results. In the absence of Hh, some Ci must enter the nucleus, as accumulation in A cells is seen in the presence of LMB. It is probably exported rapidly, since...
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no nuclear Ci is detected without LMB treatment. In the presence of Hh, more Ci enters the nucleus, but it is also rapidly exported, explaining why little nuclear accumulation is seen in untreated P cells.

Next, we studied the effect of LMB on the subcellular localization of Fu and Su(fu). dpp-lacZ was used as a marker for nuclei and the AP compartment boundary, and mycSu(fu) and mycFu were expressed in cell clones using an act5c>CD2>Gal4 transgene (Pignoni and Zipursky, 1997). Su(fu) was largely confined to the cytoplasm of untreated or LMB-treated wing imaginal discs (Fig. 5C,D). Fu was also cytoplasmic in untreated cells (Fig. 5E), but showed some accumulation in the nuclei of LMB treated cells (Fig. 5F). In contrast to Ci, the quantity of nuclear Fu did not depend on Hh exposure (data not shown). The subcellular localization of myc-tagged Cos2 was also examined. mycCos2 was mainly cytoplasmic in untreated imaginal disc cells (Fig. 5G), but partly nuclear in LMB-treated cells (Fig. 5H). As with Fu, no difference in nuclear accumulation between A and P cells could be detected. Thus, Fu and Cos2 can enter the nucleus, but the rate of entry does not change with Hh signaling.

Su(fu) prevents nuclear accumulation of Ci

A possible mechanism by which Fu stimulates and Su(fu) counteracts Ci[act] could be the promotion or impediment of nuclear Ci[act] accumulation, respectively. We compared the subcellular distribution of CiPKA in cells expressing or lacking Su(fu). Strikingly, we found that, while wild-type cells show a cytoplasmic distribution of CiPKA, this protein is mostly nuclear in Su(fu) mutant salivary gland cells (Fig. 4G,H). Identical results were obtained with wild-type Ci fused N terminally to GFP (data not shown). This suggests that Su(fu) influences the nuclear localization of Ci. We further tested the effect of Su(fu) on Ci localization by overexpressing Su(fu) with a GFP-tagged form of Ci75, which has been shown to be mainly nuclear (Aza-Blanc et al., 1997; Hepker et al., 1997, Fig. 6A). Expression of mycSu(fu) reduced the amount of GFPCi75 that accumulates in the nucleus (Fig. 6B). We also performed these experiments with full-length Ci (CiGFP). As shown before, CiGFP accumulates in LMB-treated nuclei, particularly in those exposed to Hh (Fig. 6C). Co-expression of Su(fu) in LMB-treated discs reduced the amount of nuclear CiGFP, both in A and P cells (Fig. 6D). We conclude that Su(fu) downregulates the Hh pathway by preventing nuclear accumulation of Ci[act].

**DISCUSSION**

Ci[act] and Ci[rep] are two distinct forms of Ci whose generation is strictly under the control of Hh. The mechanism by which full-length Ci is converted into Ci[act] is unknown, as are the precise events leading to the formation of Ci[rep]. Using the Ci target genes ptc, dpp and hh as diagnostic tools for the presence of Ci[act] or Ci[rep], we demonstrate that PKA and Cos2 prevent Ci[act] formation and promote the generation of Ci[rep]. The structural integrity of Fu also contributes to Ci[rep] formation. Loss of PKA activity leads to the appearance of Ci[act], an effect that can be mimicked by mutating PKA phosphorylation sites of Ci. The key finding of our present study is the discovery that, although such mutant forms of Ci are constitutively active, their activity can be further stimulated by Hh signaling. Our results indicate that this effect is mediated by Fu and counteracted by Su(fu). We suggest that Fu, rather than being involved in Ci[act] formation per se, stimulates the Hh pathway by permitting nuclear accumulation of Ci[act]. Below we describe arguments that support this proposal.
The roles of Fu and Su(fu)

Su(fu) is not involved in the formation of Ci[rep] or Ci[act], as no ectopic dpp or ptc expression was detected in Su(fu)LP clones (data not shown). Indeed, Ohlmeyer and Kalderon (1998) report no change in the amount of Ci75 in Su(fu)LP discs. Rather, Su(fu) appears to restrict the activities of Ci[act]. This is evident from the observation that Su(fu) overexpression substantially curbs the transcriptional activity of constitutively active CiPKA, and is suggestive of Su(fu) acting after Ci[act] formation. There are several ways by which Su(fu) could fulfill such a role. One possibility is that it impedes entry of Ci[act] into the nucleus. Alternatively, Su(fu) might promote nuclear export of Ci[act]. It is difficult to distinguish between these two possibilities. The observation that little Su(fu) accumulated in the nuclei of LMB-treated cells suggests that Su(fu) functions primarily in the cytoplasm and hence might exert a negative effect on Ci[act] by preventing its nuclear entry. It cannot be excluded, however, that a minor fraction of Su(fu) negatively affects the activity, stability or localisation of Ci[act] in the nucleus.

Fu, as the main regulator of Su(fu) activity, is also controlled by Hh. In fu discs, CiPKA expression leads to similar levels of ptc transcription in A and P cells but, in fu discs, CiPKA expression causes higher ptc levels in P cells. In other words, Fu enhances CiPKA activity only in Hh-exposed cells. From this, it can be concluded that Fu activity is subject to Hh control.

One puzzling aspect regarding Su(fu) is that it is dispensable for viability. Animals that lack Su(fu) protein do not exhibit Hh-independent Ci[act] activity. This paradox can be partly explained by viewing Su(fu) only as a partial inhibitor of Ci[act] activity, which exerts its function subsequent to Ci[act] formation. Other elements, discussed below, ensure tight control over the generation of Ci[act].

Fig. 7. Model in which maximal activation of Hh target genes occurs in a two-step process. Complex formation with Fu, Cos2 and microtubules serves to tether Ci to the cytoplasm and to locate Ci to the site of Slimb-dependent proteolytic processing. Hh stimulates the release of this complex from microtubules, leading to the formation of Ci[act]. The latter can still be partially inhibited through the action of Su(fu), either by suppressing nuclear import (as indicated in the diagram) or by enhancing nuclear export (not indicated). Hh stimulation of Fu kinase counteracts this effect and allows Ci[act] to accumulate in the nucleus. Nuclear Ci is rapidly exported, both in Hh receiving cells as well as in non-Hh receiving cells (see also Chen C.-H. et al., 1999).

The problem of how full-length Ci protein is converted into Ci[act] is more challenging. Fu has been implicated in this process (Ohlmeyer and Kalderon, 1998; this work), but as in the case of Su(fu), Fu kinase activity is partially dispensable in wild-type discs and entirely dispensable in animals lacking Su(fu) (Préat, 1992; Préat et al., 1993). This suggests that the Su(fu) acts subsequent to Ci[act] formation, we must conclude that the same is true for the Fu kinase. In short, we propose that the activity of the Fu kinase is only required to maximize the output of an already activated form of Ci, for example by opposing cytoplasmic tethering of Ci[act] by Su(fu). The precise mechanism of how these components act is not understood. No substrate for the Fu kinase has been identified and the significance of nuclear Fu protein is unclear.

Ci[rep] formation

Slimb, Cos2, PKA and Fu are required for the conversion of full-length Ci into the repressor form. Basal levels of dpp are transcribed in anterior slimb clones, indicating absence of Ci[rep] in these cells. No Ci[rep] could be detected by our repressor assay in cells mutant for cos2 or PKA. Moreover, we found that an intact C terminus of Fu is required to generate Ci[rep], whereas Fu kinase activity is dispensable.

Ci[act] formation

slimb mutant cells do not ectopically express ptc (N.M. and K.B., unpublished). Similar results were recently reported by Wang et al. (1999), who have also used a slimb null allele. Thus Slimb seems to play no role in the generation of Ci[act]. PKA and Cos2, in contrast, appear to be components dedicated to prevent Ci[act] formation, as cells lacking these proteins ectopically express ptc. This suggests that some of the events leading to Ci[act] include the neutralization of PKA and Cos2 activities. PKA directly phosphorylates Ci in vitro and mutations at the PKA phosphorylation sites of Ci have a profound effect on Ci activity (Chen et al., 1998; Chen Y. et al., 1999; Wang et al., 1999; Price and Kalderon, 1999; this study). Since no change in catalytic activity has been reported for PKA upon Hh signaling (Ohlmeyer and Kalderon, 1997), it is thought that other events, such as protection of Ci from phosphorylation by PKA, or dephosphorylation of Ci, may be involved in activation. Indeed, Chen C.-H. et al. (1999) provide pharmacological evidence for specific phosphatases that modulate the phosphorylation state of Ci.
The role of complex formation

We have shown that PKA and Cos2 prevent Ci[act] formation and that the same components are required for Ci[rep] formation. This observation closely links the two events. Cos2, Fu and Ci are found in a large cytoplasmic complex that is associated with microtubules (Sisson et al., 1997; Robbins et al., 1997). Fu derived from type II alleles, lacking the C-terminal portion, fails to locate to this complex (Robbins et al., 1997). Indeed, Ci[rep] is not generated in cells expressing only type II-mutant Fu protein. In addition, exposure to Hh releases Cos2 from microtubules (Robbins et al., 1997). This links Ci[rep] formation to complex formation. Together, these findings lead us to surmise that complex formation fulfills two roles: one is to tether Ci to microtubules, thereby preventing nuclear entry. The other is to localize Ci to the site of proteolytic processing for the formation of Ci[rep]. Hh signaling would promote the formation of Ci[act] by releasing this complex (or Ci) from microtubules, and as a consequence would prevent the cleavage of Ci. Upon release, Ci[act] would be subjected to Su(fu) action, possibly by cytoplasmic tethering. Stimulation of Fu kinase activity by Hh inhibits Su(fu) and enables nuclear accumulation of Ci[act] (Fig. 7). This model is consistent with the genetic data on Fu and Su(fu).

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