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Nuclear import of Cubitus interruptus is regulated by Hedgehog via a mechanism distinct from Ci stabilization and Ci activation

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SUMMARY

The Hedgehog (Hh) signal is transduced via Cubitus interruptus (Ci) to specify cell fates in the *Drosophila* wing. In the absence of Hh, the 155 kDa full-length form of Ci is cleaved into a 75 kDa repressor. Hh inhibits the proteolysis of full-length Ci and facilitates its conversion into an activator. Recently, it has been suggested that Hh promotes Ci nuclear import in tissue culture cells. We have studied the mechanism of Ci nuclear import in vivo and the relationship between nuclear import, stabilization and activation. We found that Ci rapidly translocates to the nucleus in cells close to the anteroposterior (AP) boundary and this rapid nuclear import requires Hh signaling. The nuclear import of Ci is regulated by Hh even under conditions in which Ci is fully stabilized. Furthermore, cells that exhibit Ci stabilization and rapid nuclear import do not necessarily exhibit maximal Ci activity. It has been previously shown that stabilization does not suffice for activation. Consistent with this finding, our results suggest that the mechanisms regulating nuclear import, stabilization and activation are distinct from each other. Finally, we show that *cos2* and *pka*, two molecules that have been characterized primarily as negative regulators of Ci activity, also have positive roles in the activation of Ci in response to Hh.

Key words: *hedgehog*, *cubitus interruptus*, Signaling pathway, Nuclear import, Activation, *costal-2*, *pka*, *fu*, *Drosophila melanogaster*

INTRODUCTION

hedgehog (*hh*) and its vertebrate homologues, most importantly *Sonic hedgehog* (*Shh*), play critical roles in development. In vertebrates, members of the *hh* family function in various developmental processes ranging from neural tube patterning to hair follicle formation (Echelard et al., 1993; St-Jacques et al., 1998). In *Drosophila*, *hh* is required for embryonic segmentation, for CNS development, and for the patterning of the primordia of adult appendages, the imaginal discs.

The wing imaginal disc is an ideal system for studying the Hh signaling pathway. Cells in a wing disc are subdivided into two adjacent populations that do not intermingle, the anterior and posterior "compartments" (Garcia-Bellido et al., 1973; Lawrence and Morata, 1976). *hh* is exclusively expressed in posterior compartment cells, and the Hh protein traverses the compartment boundary to regulate gene expression in the anterior compartment (Lee et al., 1992; Tabata et al., 1992; Tabata and Kornberg, 1994). *hh* has at least two major roles in the anteroposterior (AP) patterning of the wing: a long-range, indirect activity (Zecca et al., 1995) and a short-range, direct activity (Muller et al., 1997; Strigini and Cohen, 1997). The long-range activity of *hh* is mediated by one of its target genes, *decapentaplegic* (*dpp*), a member of the transforming growth factor β (TGFβ) family (reviewed by Gelbart, 1989). The short-range activity is independent of *dpp* and is essential for correct AP patterning of the medial section of the wing.

The Hh signal transduction pathway is initiated when Hh binds to its receptor Patched (Ptc), which both transduces and sequesters the Hh signal (Chen and Struhl, 1996). Ptc forms a complex with Smoothened (Smo), a serpentine transmembrane protein with features of G-protein-coupled receptors (Alcedo et al., 1996; van den Heuval and Ingham, 1996), and represses Smo activity in the absence of Hh binding (Chen and Struhl, 1998). Upon Hh binding, the repression of Smo is relieved by an as yet unknown mechanism. Through the action of a number of downstream molecules including the kinesin-like protein, Costal-2 (Cos2) (Robbins et al., 1997; Sisson et al., 1997), the serine/threonine kinase, Fused (Fu) (Sanchez-Herrero et al., 1996; Alves et al., 1998), protein kinase A (PKA) (Jiang and Struhl, 1995; Johnson et al., 1995; Lepage et al., 1995; Li et al., 1995; Pan and Rubin, 1995) and the PEST protein, Suppressor of fused [Su(fu)] (Ohlmeyer and Kalderon, 1998), the Hh signal modulates the activity of the zinc-finger transcription factor, Cubitus interruptus (Ci) (Alexandre et al., 1996; Dominguez et al., 1996; Hepker et al., 1997), thereby regulating the expression of its target genes.

Ci is the *Drosophila* homologue of the vertebrate Gli proteins (Orenic et al., 1990) and the only identified transcription factor

in the Hh signaling pathway. Given the critical role that Ci plays in the expression of Hh target genes, the mechanisms by which the Hh signal regulates Ci have been the subject of intense study, and significant progress has been made in the past several years. In the wing disc, *ci* is expressed throughout the anterior compartment (Motzny and Holmgren, 1995). The full-length Ci protein is proteolytically cleaved to generate a 75 kDa repressor form (Ci-75) in cells away from the compartment boundary, where the Hh signal is absent or of very low concentration (Aza-Blanc et al., 1997). The F-box/WD40-repeat protein, Slimb, a relative of yeast Cdc4p, which targets cell-cycle regulators for ubiquitination and subsequent proteasome degradation, is required for Ci cleavage (Jiang and Struhl, 1998). PKA and Cos2 are also required (Jiang and Struhl, 1998; Wang and Holmgren, 1999), and Ci becomes resistant to cleavage when putative PKA sites are mutated (Chen et al., 1998, 1999b; Price and Kalderon, 1999). The Hh signal stabilizes Ci by inhibiting cleavage. Hh also up-regulates the activity of Ci through a mechanism distinct from Ci stabilization (Methot and Basler, 1999, Wang et al., 1999; Wang and Holmgren, 1999), an event referred to as "Ci activation". Finally, the amount of nuclear Ci appears to be tightly controlled through a cytoplasmic/nuclear shuttling process, and it has been proposed that Hh regulates the nuclear import of Ci (Chen et al., 1999a), which contains a functional nuclear localization signal (NLS) but is primarily cytoplasmic (Wang and Holmgren, 1999).

While Ci stabilization can be monitored by staining and by western blotting, the other two aspects of Ci regulation have been hard to follow directly, and many questions remain to be answered. In spite of the suggestion that Hh accelerates the nuclear import of Ci, Ci remains predominantly cytoplasmic in cells receiving high levels of Hh in vivo (Motzny and Holmgren, 1995), as well as in tissue culture cells subjected to Hh-N treatment (except when the cells are transfected to over-express *ci*) (Chen et al., 1999a). Similarly, while nuclear export has been implicated in the regulation of Ci subcellular distribution, inhibition of nuclear export in cultured cells, with or without Hh-N treatment, does not result in dramatic changes in Ci localization (Chen et al., 1999a). Here we address several questions regarding the regulation of Ci. To what extent is Ci nuclear import regulated by Hh, and what are the mechanisms that coordinate this process? What is the significance of nuclear export? Moreover, what is the relationship between Ci nuclear import, stabilization, and activation? Although Ci stabilization alone does not suffice for Ci activation (Methot and Basler, 1999; Wang and Holmgren, 1999), could the mechanism of Ci activation be accounted for by accelerated nuclear import of stabilized Ci?

In this report we studied the mechanism of Ci nuclear import in vivo using a novel assay in which imaginal discs are cultured as whole-mount organs for short periods of time. Our results show that Hh signaling dramatically increases the rate of Ci nuclear import and that rapid nuclear export has a major role in maintaining the normal pattern of Ci subcellular distribution. The mechanism regulating Ci nuclear import is distinct and genetically separable from that regulating Ci stabilization and activation. Analysis of the roles of *cos2* and *pka* in Ci nuclear import and Ci activation reveals dual functions for *cos2* and *pka* in Ci regulation.

(For convenience, in this report "Ci" will refer to the full-length form of Ci, Ci-155, unless otherwise specified.)

MATERIALS AND METHODS

Fly stocks

cos2^{1} FRT42, *DC0^{H2} myc FRT40A* and *fu^{mH63}*, *smo^{D16} DC0^{H2} FRT40A*, *smo^{IIG26} FRT40A*, *UAS-ci(m1-3)*, *ptc^{IN}* and *ptc^{G20}* were kindly provided by T. Orenic, D. Kalderon, G. Struhl, S. Blair, R. Whittle and S, Smolik, respectively. *cos2^{1}* is an embryonic lethal, loss-of-function allele. *DC0^{H2}* is a null allele for the catalytic domain of protein kinase A. *smo^{D16}* and *smo^{IIG26}* are loss-of-function and hypomorphic alleles, respectively. *ptc^{IN}* is a null and *ptc^{G20}* is a hypomorphic allele. *ci(m1-3)* encodes a form of Ci in which the PKA sites at Ser-838, Ser-856 and Ser-892 have been mutated (Chen et al., 2000). *fu^{94}* is a strong hypomorphic allele.

Generation of *ptc* hypomorphic discs

ptc hypomorphic discs are of the genotype *ptc^{IN}/ptc^{G20}*.

Generation of somatic clones

Somatic recombinant clones were generated by means of the FLP FRT technique (Xu and Rubin, 1993). Larvae were heat-shocked for 1 hour at 37°C, 48-72 hours after egg laying. Genotypes of the larvae were as follows:

smo clones: *y hsflp; smo^{IIG26} FRT40A/myc FRT40A*
pka clones: *y hsflp; DC0^{H2} myc FRT40A/FRT40A*
pka clones, *4bslacZ: y hsflp; DC0^{H2} myc FRT40A/FRT40A; 4bslacZ/+*
pka smo clones: *y hsflp; smo^{D16} DC0^{H2} FRT40A/myc FRT40A*
cos2 clones: *y hsflp; cos2^{1} FRT42/myc FRT42*
cos2 clones, *4bslacZ*: *y hsflp; cos2^{1} FRT42/myc FRT42; 4bslacZ/+*

Immunohistochemistry

Imaginal discs were examined by deconvoluting microscopy. Antibodies used were as follows: anti-Ci, rat monoclonal 2A1 (1:2); anti-β-galactosidase, rabbit polyclonal (1:2000); anti-Ptc, mouse monoclonal ascites (gift from I. Guerrero) (1:1000); and all fluorescent secondary antibodies (1:200, Jackson ImmunoResearch Labs).

Imaginal disc culture

Wing imaginal discs from mid-third instar larvae were incubated at 25°C in *cl-8* cell medium for up to 3 hours prior to staining. For treatment with leptomycin B (LMB), discs were cultured in medium containing 50 ng/ml LMB (experimental discs) or an equal volume of solvent (ethanol) (control discs). For treatment with MG132, discs were cultured in medium containing 10 μM MG132 (experimental discs) or an equal volume of solvent (DMSO) (control discs).

RESULTS

Rapid nuclear/cytoplasmic shuttling of Ci in cells along the AP boundary

In *Drosophila* wing imaginal discs, *ci* is expressed throughout the anterior compartment. In cells away from the boundary, the level of full-length Ci is low due to proteolytic cleavage. In cells along the AP compartment boundary, Hh signaling inhibits Ci cleavage, creating a stripe of elevated Ci. The Hh target gene *dpp* is expressed in a stripe roughly corresponding to the high-level Ci stripe, and *ptc* is expressed at high levels in the 2-3 cells immediately adjacent to the AP boundary (Fig. 1A). Ci contains a functional NLS but is primarily cytoplasmic throughout its expression domain (Fig. 1B). We examined the subcellular distribution of Ci in discs treated with leptomycin B (LMB), a potent inhibitor of protein nuclear export (Fukuda et al., 1997). Within 1 hour, LMB-treated discs exhibit nuclear

accumulation of Ci in a stripe of cells along the AP boundary (Fig. 1C), suggesting that within these cells, Ci is normally rapidly shuttled between the nucleus and the cytoplasm. Ci in cells away from the boundary remains cytoplasmic, and there is no visible change in the pattern of Ci subcellular distribution in these cells even after 3 hours of treatment. In LMB-treated discs, the region of nuclear Ci correlates with that of Ci stabilization. To determine whether the absence of nuclear Ci in cells away from the compartment boundary is caused by cleavage, discs were simultaneously treated with LMB and MG132, an inhibitor of proteasome activity. In discs co-treated with LMB and MG132, Ci is stabilized throughout the anterior compartment but only accumulates in the nuclei along the compartment boundary (Fig. 1D-F). Taken together, these results show that the rate of Ci nuclear import is significantly different between cells along the boundary and those away from the boundary and furthermore, this difference is not a simple reflection of the difference in Ci protein levels. Although Ci is uniformly cytoplasmic in untreated discs, its cytoplasmic distribution is maintained by different mechanisms in different regions of the disc. On the one hand, dynamic import takes place in cells along the boundary but is masked by rapid nuclear export. On the other hand, away from the boundary Ci either does not translocate to the nucleus or translocates at a very low rate.

Regulation of Ci nuclear import is Hh dependent

To test whether the nuclear import of Ci is regulated by Hh, we analyzed mutations that either mimic or block Hh signaling. In LMB-treated *ptc* hypomorphic wings, Ci is nuclear throughout the anterior compartment (Fig. 1G). Conversely, *smo* loss-of-function clones, which cannot transduce the Hh signal (Alcedo et al., 1996; Chen and Struhl, 1996; van den Heuval and Ingham, 1996), do not accumulate nuclear Ci upon LMB treatment (Fig. 1H). Therefore, the rapid nuclear import of Ci is dependent on Hh signaling.

pka is dispensable for the regulation of Ci nuclear import

Previous studies have shown that *pka* has a negative role in the expression of *hh*/*ci* target genes. Mutations in *pka* are associated with disc overgrowth and duplication, signs of up-regulated Ci activity. The level of full-length Ci is elevated in *pka* loss-of-function clones and these clones ectopically express *ptc* and *dpp* (Jiang and Struhl, 1995; Johnson et al., 1995; Lepage et al., 1995; Li et al., 1995; Pan and Rubin, 1995). Subsequent work demonstrated that PKA activity is required for the cleavage of Ci (Chen et al., 1998; Jiang and Struhl, 1998; Chen et al., 1999b; Price and Kalderon, 1999). We studied the role of *pka* in regulating Ci nuclear import by treating discs carrying *pka* clones with LMB. Without LMB treatment, Ci is primarily cytoplasmic in all clones (Johnson et al., 1995) as well as in all wild-type cells. After LMB treatment, Ci is nuclear within *pka* clones along the boundary and cytoplasmic within those away from the boundary (Fig. 2A-C), suggesting that in *pka* mutant cells, the regulation of Ci nuclear import is unaltered. To further demonstrate that Ci nuclear import and stabilization are genetically separated in *pka* clones, *pka smo* double mutant clones were generated along the compartment boundary. Unlike in *pka* single mutant

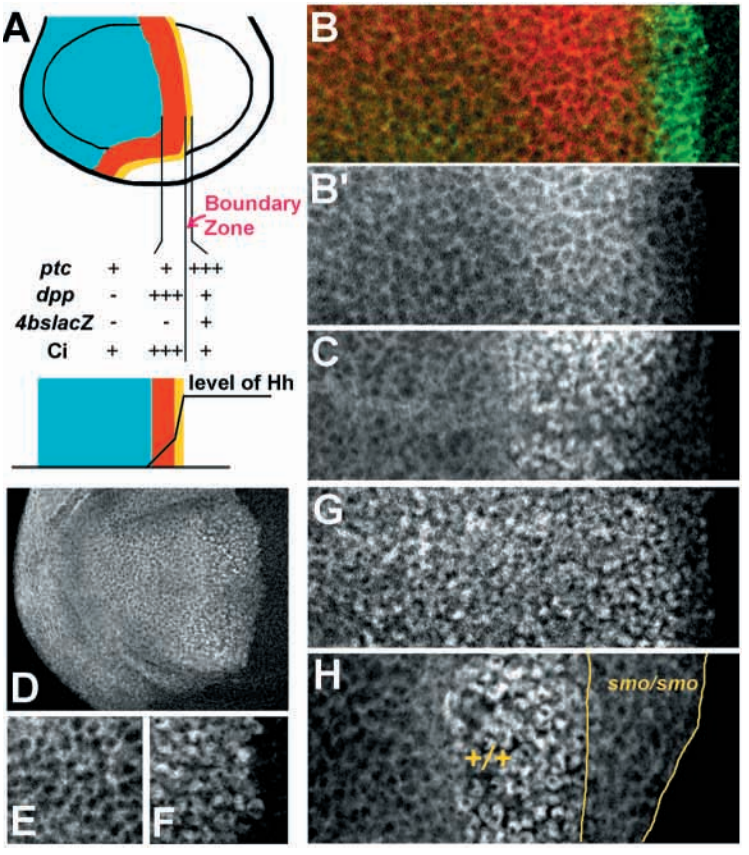

Fig. 1. Regulation of Ci nuclear import by Hh. Ci stainings were done using the monoclonal antibody 2A1 which recognizes full-length Ci and does not detect the cleaved Ci-repressor form. (A) A diagram showing the expression domains of *hh* targets and the speculated change of Hh concentration along the AP axis. In the Boundary Zone (yellow) immediately adjacent to the posterior compartment, Ci is "activated" and causes high level expression of *ptc*. Just anterior to the Boundary Zone (orange), Ci is not activated, but it is stabilized and imported to the nucleus, which is sufficient for the expression of *dpp*. (B) A wild-type wing disc doubly stained for Ci (red) and Ptc (green). B' shows Ci staining alone for the same disc. Ci is primarily cytoplasmic throughout the anterior compartment. (C) A wild-type disc treated with LMB for 1 hour. Ci accumulates in the nuclei of cells along the boundary and remains cytoplasmic away from the boundary. (D) A wild-type disc co-treated with MG132 and LMB for 2 hours. Ci accumulates to high levels throughout the anterior compartment, but is cytoplasmic in cells away from the AP boundary (E) and nuclear only in those along the boundary (F). (G) An LMB-treated *ptc* hypomorphic disc. Ci is nuclear throughout the anterior compartment. (H) A disc carrying *smo* clones co-treated with LMB and MG132 for 1 hour. Note that *smo* is required for the maintenance of the AP lineage restriction. Clones of anterior origin, if located along the AP boundary, extend into the anatomically posterior territory and can be followed by their continued expression of *ci* (Blair and Ralston, 1997; Rodriguez and Basler, 1997). Only part of a clone (outlined) is shown in this highly magnified image. Ci is cytoplasmic within the clone and nuclear in wild-type sister cells close to the AP boundary.

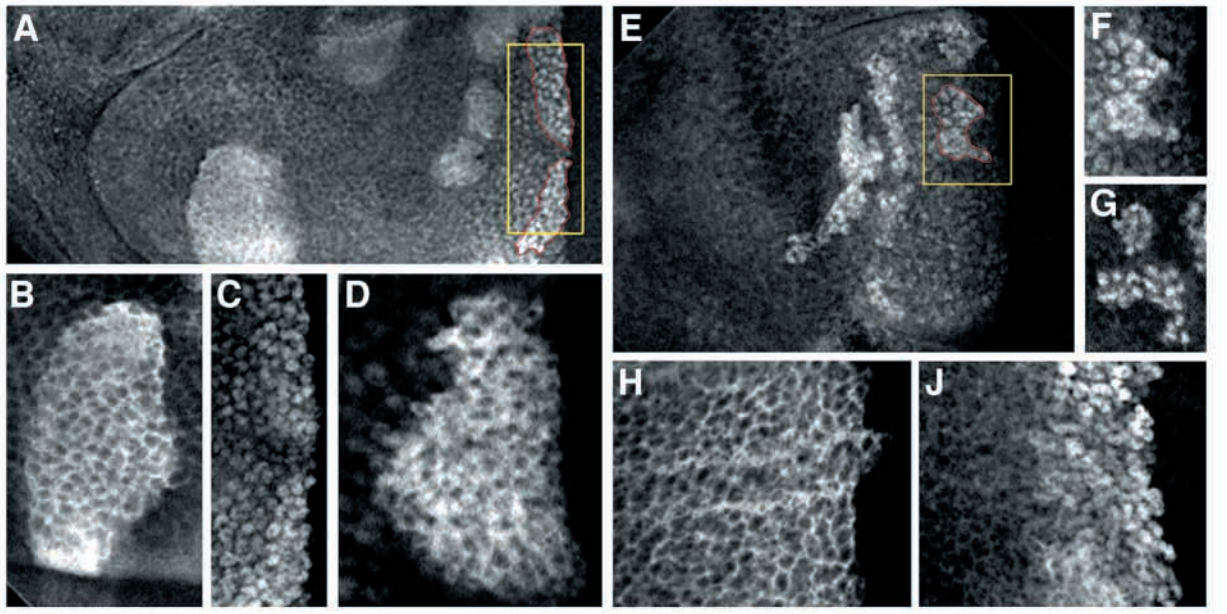

Fig. 2. Role of *pka*, *cos2* and *fu* in Ci nuclear import. All the discs shown were treated with LMB and stained for full-length Ci. *pka* and *cos2* clones have been shown to exhibit cell-autonomous elevation of Ci protein levels (Johnson et al., 1995; Sisson et al., 1997) and are thus identified in these images. (A-C) Discs carrying *pka* clones. Ci is cytoplasmic in clones away from the boundary (B) and nuclear in those along the boundary (C; high-magnification image of the region outlined in A). (D) A *smo pka* clone along the boundary. Ci is cytoplasmic, suggesting that the nuclear accumulation of Ci in *pka* boundary clones is still dependent on Hh signaling. (E-G) Discs carrying *cos2* clones. Ci is nuclear in clones along the boundary (F; high-magnification image of the region outlined in E) as well as in those away from the boundary (G). (H) A fu^{94} disc. Ci remains cytoplasmic along the boundary. fu^{1}, fu^{mH63} (class I) and fu^{RX15} (class II) discs behave similarly though with slightly weaker phenotypes (data not shown). (G) A fu^{94} disc in which *ptcGAL4* was used to direct *UAS-ci* expression along the boundary. Nuclear accumulation is rescued in cells with high levels of Ci.

clones at similar locations, Ci failed to accumulate in nuclei in the double mutant clones (compare Fig. 2D with 2C). Thus, *pka* clones exhibit cell-autonomous Ci stabilization but Hh-regulated Ci nuclear import.

cos2 negatively regulates Ci nuclear import

Another molecule that negatively regulates Ci stability and *hh* target gene expression is *cos2* (Sisson et al., 1997; Wang and Holmgren, 1999). To directly test the role of *cos2* in Ci nuclear import, discs carrying *cos2* clones were treated with LMB. All clones exhibit cell-autonomous nuclear accumulation of Ci regardless of the clone's distance from the AP boundary (Fig. 2E-G). *cos2* encodes a kinesin-like molecule that is part of a multi-protein complex including Ci (Robbins et al., 1997; Sisson et al., 1997; Monnier et al., 1998). Over-expression of *cos2* blocks nuclear entry of Ci in tissue culture cells (Chen et al., 1999), and it has been proposed that Cos2 regulates the microtubule-association of the complex and consequently the ability of Ci to translocate to the nucleus (Robbins et al., 1997). Our results are consistent with this model.

Rapid Ci nuclear import requires *fu*

Discs mutant for *fu* show signs of compromised Ci activity, including fusion between wing veins 3 and 4, lack of late *en* expression in the anterior compartment, and diffuse *ptc* expression at lower levels. Examination of these discs after LMB treatment reveals a lack of Ci nuclear accumulation (Fig. 2H), suggesting that *fu* function is required for rapid Ci nuclear import. It has been shown previously that overexpression of *ci* can rescue the wing vein phenotype in *fu* mutants (Alves et al., 1998). Consistent with this finding, the Ci nuclear import phenotype is also partially rescued in *fu* discs in which a *UAS-ci* transgene was over-expressed along the AP boundary via *ptcGAL4* (Fig. 2J).

Ci activation requires *cos2*

We have previously studied the role of *cos2* in Hh signal transduction by examining the expression of *ptc* in *cos2* clones. High level *ptc* expression is dependent on Hh signaling and is normally found only in the Boundary Zone, namely the 2-3 rows of cells immediately anterior to the compartment boundary. Although *cos2* clones away from the boundary can ectopically induce *ptc* expression (Wang and Holmgren, 1999), a small number of such clones do not express *ptc* (data not shown). A striking variability in the expression of *ptc* was observed for *cos2* clones abutting the boundary: approximately 50% of *cos2* clones overlapping the Boundary Zone disrupt the wild-type high-level Ptc stripe (Fig. 3A-C). It is not known why *cos2* activity is more critical along the boundary than away from the boundary. Nonetheless, this result suggests that *cos2* is required for Ci to become fully active in the Boundary Zone in response to a high level of Hh signaling. A requirement for *cos2* is more evident when we assayed an in vivo reporter of Ci activity, *4bslacZ*, whose enhancer element contains only four Ci binding sites and four Scalloped binding sites (Hepker et al., 1999) and whose response to Ci exhibits a more stringent requirement for Hh signaling than that of *ptc* (Wang and Holmgren, 1999). Not only do *cos2* clones away from the

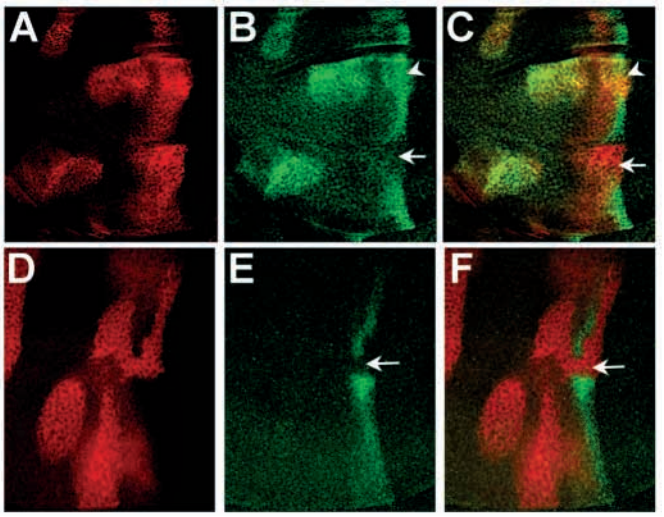

Fig. 3. *cos2* is required for proper expression of *ptc* and *4bslacZ*. Ci (red) was stained with 2A1. *cos2* clones were identified by elevated Ci protein levels. (A-C) A wing disc carrying two *cos2* clones along the boundary. One boundary clone (arrow) disrupts the endogenous *ptc* expression (green) while the other (arrowhead) ectopically express *ptc*. (D-F) A *cos2* clone abutting the boundary (arrow) disrupts the wild-type expression of *4bslacZ* (green). C and F are merged images of A,B and D,E respectively.

boundary fail to ectopically induce *4bslacZ*, but clones abutting the boundary invariably disrupt the wild-type *4bslacZ* stripe (Fig. 3D-F). Given the cell-autonomous stabilization and nuclear import of Ci in *cos2* clones, we infer that the disruption of *ptc* and *4bslacZ* expression in such clones is due to a lack of Ci activation.

Ci activation requires phosphorylation by PKA

The role of *pka* in Ci activation was assayed in the posterior compartment of discs, in which all cells are exposed to a high level of Hh ligand. An actin promoter was used to drive a low level of *ci* expression throughout the discs, as previously described (Wang and Holmgren, 1999). In discs with ubiquitous *ci*, high-level *ptc* expression is observed in the posterior compartment cells (P cells) but not in the anterior compartment cells (A cells) (Alexandre et al., 1996; Hepker et al., 1997; Methot and Basler, 1999). *pka* mutant P cells in this background do not express *ptc* (Fig. 4A-C), indicating that the loss of *pka* function disrupts Hh signal transduction and compromises maximal Ci activity. Consistent with this result, loss of *pka* function also disrupts *4bslacZ* expression in the Boundary Zone (Fig. 4D-F).

PKA has the potential to phosphorylate many substrates. While the loss of *ptc* and *4bslacZ* expression in *pka* clones could reflect a disruption of Ci activity, it could also be due to negative effects on other proteins that bind these enhancers. To distinguish between these two possibilities, we compared wild-type Ci and a PKA site-mutant form of Ci, Ci(m1-3), for their abilities to induce ectopic *4bslacZ* in the posterior compartment. Wild-type Ci exhibits a sensitive response to Hh and, at a modest protein level, induces robust *4bslacZ* expression in the posterior compartment but not in the anterior (Wang and Holmgren, 1999; Fig. 4G). In contrast, Ci(m1-3) shows no response to Hh and does not induce *4bslacZ* in either compartment (Fig. 3I,J). Thus, the integrity of specific PKA sites within Ci is essential for it to respond to high levels of Hh and become fully active. Since *pka* is dispensable for the acceleration of Ci nuclear import in Hh-receiving cells (see Fig. 2C), the requirement of PKA phosphorylation for maximal Ci activity presumably reflects a requirement for Ci activation.

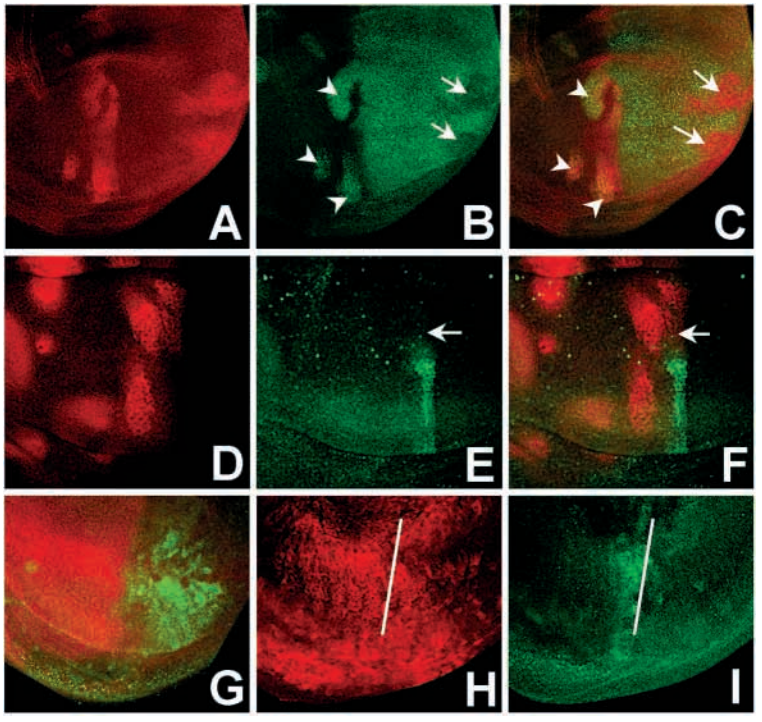

Fig. 4. An essential and direct role of *pka* in Ci activation. Ci (red) was stained with 2A1. *pka* clones were identified by elevated Ci protein levels. (A-C) *pka* clones in a disc that ubiquitously express *ci*. *ptc* (green) is expressed in wild-type P cells but not in posterior *pka* clones (arrows). Note that the anterior *pka* clones do induce ectopic *ptc* (arrowheads) as reported (Li et al., 1995). The difference between A and P cells may be due to the expression of *engrailed* (*en*) in P cells. En has a repressing effect on the *ptc* promoter (Maschat et al., 1998) which could make maximal Ci activity more critical in the P compartment. (D-F) A *pka* clone abutting the boundary (arrow) disrupts the wild-type expression of *4bslacZ* (green). (G-I) Comparison between the abilities of wild-type Ci and a PKA site-mutant form of Ci to induce ectopic *4bslacZ* expression (green). (G) Wild-type *ci*, when expressed ubiquitously, induces robust expression of *4bslacZ* (green) in the posterior compartment but not in the anterior. (H,I) A disc in which *ci(m1-3)* is expressed throughout the wing pouch via *71BGAL4* (Brand and Perrimon, 1993). *4bslacZ* (green) is not ectopically induced in either compartment (I). White bars mark the compartment boundary.

DISCUSSION

Regulation of Ci nuclear import by Hh

The predominantly cytoplasmic localization of Ci has always been intriguing because Ci has a role in the nucleus as a transcription factor. It has been speculated that Hh may play a role in regulating the nuclear import of Ci. However, there is no significant difference in the subcellular distribution of Ci between cells that receive Hh and those that do not, either in vivo (Motzny and Holmgren, 1995) or in vitro (Chen et al., 1999b). Quantitative studies in tissue culture cells show that the rate of Ci nuclear import is increased to a certain extent in the presence of Hh. However, this increase was not enough to cause visible shifts in Ci staining pattern, and accumulation of Ci can only be seen in cells that overexpress *ci* (Chen et al., 1999a). Importantly, the same study suggests that one of the factors regulating the subcellular distribution of Ci is nuclear export, which can be inhibited by treating the cells with LMB. In order to analyze the nuclear import of Ci in vivo, we subjected imaginal discs to whole-mount organ culture experiments, which allowed us to perform studies in different genetic backgrounds and under conditions that preserve the spatial context of Hh signaling. Because imaginal discs are sacs with only two layers of epidermal cells, drugs that inhibit cellular processes work as well in this context as on tissue culture cells. When protein export is blocked with LMB, the amount of Ci peptides that enter the nucleus can be monitored, and we observe a remarkable difference in cells close to and those away from the AP boundary. Ci accumulates in the nucleus only in cells close to the boundary (Fig. 1C), and this has been further shown to be Hh dependent, since mutations in *smo* prevent nuclear accumulation of Ci (Figs 1H, 2D). Unlike the situation in *cl-8* cells, the rate of Ci nuclear import in the presence of Hh is very rapid in vivo, and the subcellular distribution of Ci shifts from predominantly cytoplasmic to predominantly nuclear within as short a time as half an hour of treatment. The rate difference of Ci nuclear import in cells receiving and not receiving Hh is also significant. The cytoplasmic localization of Ci is unchanged in cells away from the boundary after three hours of treatment (data not shown). From these results, we conclude that the rate of Ci nuclear import is dramatically increased by Hh signaling in vivo. The fact that tissue culture cells do not have a similarly strong response to Hh signaling may reflect the absence of certain essential molecules. It is worth pointing out that although we could not observe Ci nuclear accumulation in cells away from the boundary, in cultured cells Ci does translocate to the nucleus at a low rate in the absence of Hh.

The negative control on Ci nuclear import is saturable

Cytoplasmic tethering has been proposed as a mechanism for regulating the subcellular distribution of Ci, and Cos2 is thought to mediate the interaction of the Ci/Cos2/Fu/Su(fu) complex with the cytoskeleton (Robbins et al., 1997). In response to Hh signaling, the whole complex was found to dissociate from the cytoskeleton, though it is as yet unclear whether the whole complex or Ci alone translocates to the nucleus. In tissue culture cells, over-expression of *ci* leads to increased nuclear import, which can be blocked by co-expression of *cos2* (Chen et al., 1999a). These results suggest that (1) a stoichiometric interaction between Cos2 and Ci is important for cytoplasmic tethering; and (2) the negative regulation provided by Cos2 is saturable. *fu* is normally required for the rapid Ci nuclear import along the AP boundary, but this requirement can also be circumvented by *ci* over-expression, suggesting that once the cytoplasmic tethering system is saturated, Hh signaling is no longer required for Ci nuclear import. A diagram of this model of Ci nuclear import regulation is given in Fig. 5A.

The mechanism of Ci nuclear import is distinct from that of Ci stabilization

Although the domain of rapid Ci nuclear import coincides with that of Ci stabilization in wild-type discs (Fig. 1C), the two events appear to be regulated through different mechanisms. Two lines of evidence suggest that the nuclear import of Ci is regulated even under conditions in which Ci is fully stabilized. The difference in import rates between cells away from and those close to the AP boundary persists when proteasome activity is blocked and Ci protein level is uniform (Fig. 1D-F). Furthermore, while Ci is fully stabilized in all *pka* clones, rapid nuclear import takes place only in those clones that are close to the AP boundary and receive Hh (Fig. 2C,D). We consider the latter result significant because it represents the first example in which different aspects of Ci metabolism have been genetically separated. The identification of mutant backgrounds which disrupt one aspect of Ci regulation but not others will be particularly useful in elucidating the mechanisms of Hh signal transduction.

Hh activates Ci through a mechanism other than nuclear import of stabilized Ci

It has been shown that Hh signaling up-regulates the activity of Ci through a process distinct from Ci stabilization, which is itself a Hh-induced event (Methot and Basler, 1999; Wang et al., 1999; Wang and Holmgren, 1999). When Hh-regulated nuclear import is taken into consideration, the question arises whether nuclear import on its own or combined with Ci stabilization could account for the mechanism of Ci activation. A prerequisite to answering this question is to define Ci activation. The activated form of Ci has not been characterized and at present cannot be followed. What can be followed is the apparent activity of Ci, which is assayed by the expression of Ci targets, including endogenous targets such as *ptc* and reporter constructs such as *4bslacZ*. *ptc* is expressed throughout the anterior compartment, but only the elevated expression in the Boundary Zone is Ci dependent (Methot and Basler, 1999). *4bslacZ* is expressed only in the Boundary Zone in wild-type discs (Hepker et al., 1999; Wang and Holmgren, 1999). Notice that the domains of Ci nuclear import and stabilization extend into the anterior compartment as far as 8-9 cells from the AP boundary, but only a subset of these cells – those in the Boundary Zone – express *ptc* and *4bslacZ*. This observation strongly suggests that in the Boundary Zone, a high level of Hh signaling elicits an activating event(s) that is distinct from Ci stabilization or nuclear import and is necessary for Ci to reach its maximal activity. The mosaic analysis of *cos2* and *pka* mutants is consistent with such a model. In *cos2* clones, Ci is stabilized and rapidly translocated into the nucleus, but the disruption of *ptc* and *4bslacZ* expression along the boundary suggests that Ci activation is blocked in the mutant cells.

Similarly, *pka* clones along the boundary lose expression of *4bslacZ*, even though these clones exhibit cell-autonomous Ci stabilization and nuclear import (Fig. 2C). Based on results described here and on those from previous studies (Methot and Basler, 1999; Wang et al., 1999), we conclude that Ci stabilization, nuclear import and activation are each regulated through a distinct mechanism and cannot substitute for each other.

A central role for *cos2* in the regulation of Ci

Our data suggest a central role for *cos2* in the regulation of Ci (Fig. 5A,B). It functions not only in Ci stability (Sisson et al., 1997; Wang and Holmgren, 1999), but also in Ci nuclear import and activation (Figs 2E-G, 3A-F). Studies in mammalian cells show that the cytoskeleton plays important roles in mediating fast and precise protein-protein interactions (Torres and Coates, 1999), and this is likely to be true in *Drosophila* as well. Given the close association of Cos2 with the cytoskeleton, it may control the association of Ci with various modulators and its intracellular movements, including its nuclear translocation. Two other components of the Hh signaling pathway, Fu and Su(fu), have been found in close association with Cos2 and Ci (Preat et al., 1993; Robbins et al., 1997; Sisson et al., 1997; Monnier et al., 1998). Thus, this complex could serve as a platform for regulating many aspects of Ci function (Fig. 5B).

Dual role for *pka* in the regulation of Ci

Previous work has suggested that *pka* regulation of Ci is complex. In embryos, *pka* appears to have both negative and positive effects on Ci function (Ohlmeyer and Kalderon, 1997). The situation is equally complex in the imaginal discs. In loss-of-function *pka* clones, Ci cleavage is blocked and the Hh target genes *ptc* and *dpp* are ectopically expressed (Jiang and Struhl, 1995; Lepage et al., 1995; Li et al., 1995; Pan and Rubin, 1995). Reciprocal experiments, in which a constitutively active form of PKA is expressed, have given different results depending upon the level of expression. At modest levels, *pka* expression has no effect (Jiang and Struhl, 1995) while at very high levels, *pka* can block the expression of *hh* target genes (Wang et al., 1999). The results from modest level *pka* expression would suggest that *pka* activity is a necessary precondition for proper Hh regulation but does not itself mediate the Hh signal. The high level *pka* expression experiments could be interpreted to implicate *pka* as a component of the Hh signaling transduction cascade. One concern with these latter experiments is that PKA at very high levels could be expected to phosphorylate many targets and have pleiotropic effects.

Our results suggest that *pka* is required for two aspects of Ci function, its previously defined role in cleavage and a second role in activation (Fig. 5B). Experiments showing that *4bslacZ* is not induced by a mutant form of Ci lacking three potential PKA phosphorylation sites strongly implicate the direct phosphorylation of Ci by PKA in the process of activation. These studies do not distinguish whether the role of *pka* in activation is instructive (as a component of the Hh signaling cascade) or permissive (establishing a necessary precondition for proper Hh regulation). Given that there is little evidence for Hh regulation of PKA activity, we at present favor the view that *pka* is permissive (Fig. 5B).

Having PKA regulate both the activation and cleavage of Ci may provide a check on inappropriate activation of Ci. It could be imagined that Ci proteins are occasionally activated away

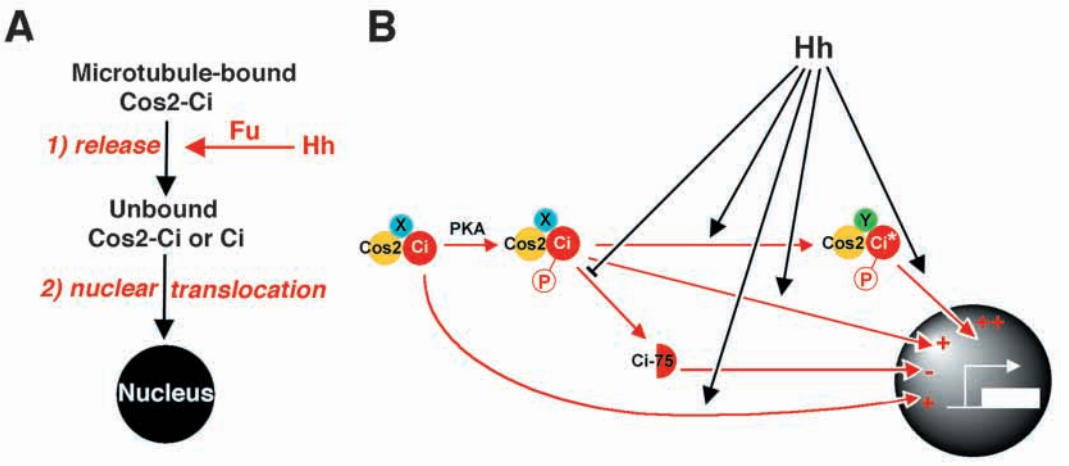

Fig. 5. Models of Ci regulation. (A) A two-step model for the regulation of Ci subcellular distribution. The first step involves the complex containing Ci, or eventually Ci itself, being released from cytoplasmic tethering forces. Hh regulates the release step through Fu. The cytoplasmic tethering system appears to be saturable. In the second step, un-tethered Ci is translocated to the nucleus via the function of its NLS. The existing evidence suggests that this step does not require Hh signaling. (B) A model summarizing the multiple events that regulate Ci. The black sphere represents the nucleus where transcription factors regulate gene expression. While all forms of full-length Ci are transcriptional activators (the plus signs), the activated form of Ci (Ci*) exhibits the highest potency on *ptc* or *4bslacZ* enhancers. All full-length forms possess the ability to enter the nucleus, but nuclear import is inhibited by Cos2 in the absence of Hh. The cleaved form of Ci, Ci-75, freely translocates to the nucleus and has a negative effect on the expression of *ci* target genes (the minus sign). Based on previous findings and results presented in this report, we propose that PKA phosphorylation of Ci is a prerequisite for both Ci cleavage and activation. To explain the diverse role of Cos2 in Ci regulation, we also propose that depending on the levels of Hh signaling, Cos2 associates, either directly or indirectly, with different factors or the same factors in different modification states (represented here by X and Y) which modulate the activity of Ci.

from the compartment boundary. Such inappropriate activation could have deleterious consequences on imaginal disc patterning. However, in the absence of Hh signaling, PKA phosphorylation of Ci will target any inappropriately activated Ci molecules to the proteasome.

Expression of *hh* target genes – readout of Ci regulation and beyond

The expression of *hh* target genes has always been used as a readout of Hh signaling and Ci regulation. The interpretation of the expression pattern, however, is not always straightforward. For example, *pka* clones away from the AP boundary ectopically express *dpp* and *ptc*, yet the results presented in this paper suggest that Ci in these clones is neither activated nor translocated to the nucleus at a high rate. Why, then, is Ci stabilization in *pka* clones sufficient to induce ectopic *ptc*? In addressing this question, several points need to be taken into consideration.

In *pka* clones, the protein level of Ci is elevated due to the lack of cleavage. Although the majority of Ci stays tethered in the cytoplasm in the absence of Hh signal, the cytoplasmic tethering sites for Ci could be saturated with some Ci molecules translocating to the nucleus where it can act as a transcriptional activator. In stainings, the nuclear import of these molecules would be masked by the preponderance of cytoplasmic Ci.

Secondly, the expression of a gene is affected by its enhancer structure and all the proteins that interact with the enhancer. A 400 bp element from the *dpp* enhancer harbors binding sites for multiple factors, and activation of this element has been shown to depend upon the synergistic action of Ci with other DNA-binding proteins (Hepker et al., 1999). The whole *dpp* disc enhancer is 10 kb in length and probably contains many such elements. The *ptc* and *en* enhancers are likely to be equally complex. The behavior of these enhancers is certainly not due to the action of Ci alone, and it can be imagined that mutation in *pka* may affect the activity of factors involved in their regulation. Our results with *4bslacZ* are the most straightforward and this probably reflects the simplicity of this artificial enhancer.

Finally, it is likely that what we call “stabilized”, “activated” and “nuclear imported” Ci each consists of a variety of isoforms with distinct properties. The overall activity of these molecules is not always easy to predict. For instance, Ci is stabilized in both *pka* and *slimb* clones, but is barely active in the latter (Wang et al., 1999). A better understanding of the regulation of Ci awaits further genetic, biochemical and cell biological studies.

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