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Proteolysis That Is Inhibited by Hedgehog Targets Cubitus interruptus Protein to the Nucleus and Converts It to a Repressor

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Summary

Cell–cell communication at anterior/posterior compartment borders in Drosophila involves Hedgehog (Hh), a protein secreted by posterior cells, and Cubitus interruptus (Ci), a protein in the Hh response pathway in anterior cells. Although Ci is thought to have roles as a transcription factor repressing *hh* expression and activating target genes, it localizes in the cytoplasm of anterior cells. We report here the identification of a domain that tethers Ci in the cytoplasm and show that in some anterior cells, Ci is cleaved to generate a form that lacks the tethering domain. This form translocates to the nucleus where it represses *hh* and other target genes. Hh inhibits proteolysis of Ci, and we suggest that this inhibition leads to the observed patterns of expression of key target genes at the compartment border.

Introduction

Anterior/posterior (A/P) compartment borders orchestrate interactions between key regulatory genes to control the growth and development of Drosophila imaginal discs. In developing wing imaginal discs, for instance, patterns form as a function of distance from the A/P compartment border (Crick and Lawrence, 1975; Lawrence and Morata, 1976; Tabata et al., 1995; Zecca et al., 1995), suggesting that at least in this organ, the compartment border acts as a classical organizer. A/P compartment borders form at the juxtaposition of two distinct populations of cells—P compartment cells that express *engrailed* (*en*) and *hedgehog* (*hh*), and A compartment cells that express *cubitus interruptus* (*ci*) and other A-specific genes such as *patched* (*ptc*) and *decapentaplegic* (*dpp*). These two cell populations function as separate units of growth and patterning (Lawrence and Morata, 1976; Tabata et al., 1995), but they are also critically dependent upon each other.

For example, A cells at the A/P compartment borders of imaginal discs express *dpp* (Raftery et al., 1991), and the Dpp protein secreted by these border cells is essential to the growth of both compartments (Spencer et al., 1982). *dpp* expression is itself dependent upon a Hh signal that emanates from P cells, as well as on various functions in the anterior cells that transduce the Hh response. These A-specific functions include Ptc and Smo, two membrane proteins that are thought to constitute the Hh receptor (Alcedo et al., 1996; Chen and Struhl, 1996; Marigo et al., 1996; Stone et al., 1996); Fu, a putative serine/threonine protein kinase (Préat et al., 1990); Su(fu), a protein without known function (Pham et al., 1995); Cos-2, a protein related to the kinesin motor (Sisson et al., 1997); protein kinase A (PKA; Kalderon and Rubin, 1988); and Ci, a putative zinc finger–containing transcription factor (Eaton and Kornberg, 1990; Orenic et al., 1990).

In this work, we focus on the role of *ci* in the response of A compartment cells to the Hh signal. Genetic and molecular analysis has revealed that Ci functions downstream of both Fu and Cos-2 (Motzny and Holmgren, 1995; Alexandre et al., 1996; Sanchez-Herrero et al., 1996) and that *ci* function is required to activate transcription of *wingless* (*wg*), *ptc*, and *dpp*, and to repress *hh* transcription (Alexandre et al., 1996; Domínguez et al., 1996; Hepker et al., 1997). In addition, levels of Ptc and Ci proteins are elevated in cells near the A/P compartment border (Phillips et al., 1990; Motzny and Holmgren, 1995), and the Fu protein itself is phosphorylated (Therond et al., 1993). If the Hh signal is not transmitted, levels of *ptc* expression and apparent levels of Ci protein are not elevated, *dpp* is not transcribed, and Fu protein is not phosphorylated (Tabata et al., 1992; Basler and Struhl, 1994; Capdevila et al., 1994; Felsenfeld and Kennison, 1995; Sanicola et al., 1995; Thérond et al., 1996).

Molecular analysis has identified a single *ci* transcription unit whose conceptual translation predicts a protein that has a domain with five presumptive zinc fingers and significant sequence identity with Tra-1 of C. elegans (Zarkower and Hodgkin, 1992) and the vertebrate Gli proteins (Kinzler et al., 1987; Ruppert et al., 1990). Alexandre et al. obtained evidence that Ci acts as a transcription factor, finding that Ci can recognize GLI consensus binding sites in vivo and can directly or indirectly induce *hh* target genes (Alexandre et al., 1996). Surprisingly, cytological studies failed to detect significant levels of Ci protein in nuclei, either near the compartment border or elsewhere (Motzny and Holmgren, 1995), so the mechanism of Ci action remains uncertain.

This study was undertaken to investigate how Ci functions in Hh signal transduction. We characterized the Ci protein and found that it exists in at least two forms, one full-length and the other truncated, and that these forms differ in activity and subcellular location. Processing of the full-length protein is inhibited by Hh, an observation that represents the first direct evidence that Ci transduces the Hh signal. These results may also be relevant to the way in which the different vertebrate Gli proteins are regulated.

Results

The 155 kDa and 75 kDa Forms of Ci Protein Are Present in Embryos and Imaginal Discs

Although expression of *ci* mRNA is uniform in A compartments of embryos and imaginal discs, Ci protein levels

*This paper is dedicated to the memory of M.-P. Laget, deceased November 3, 1996.

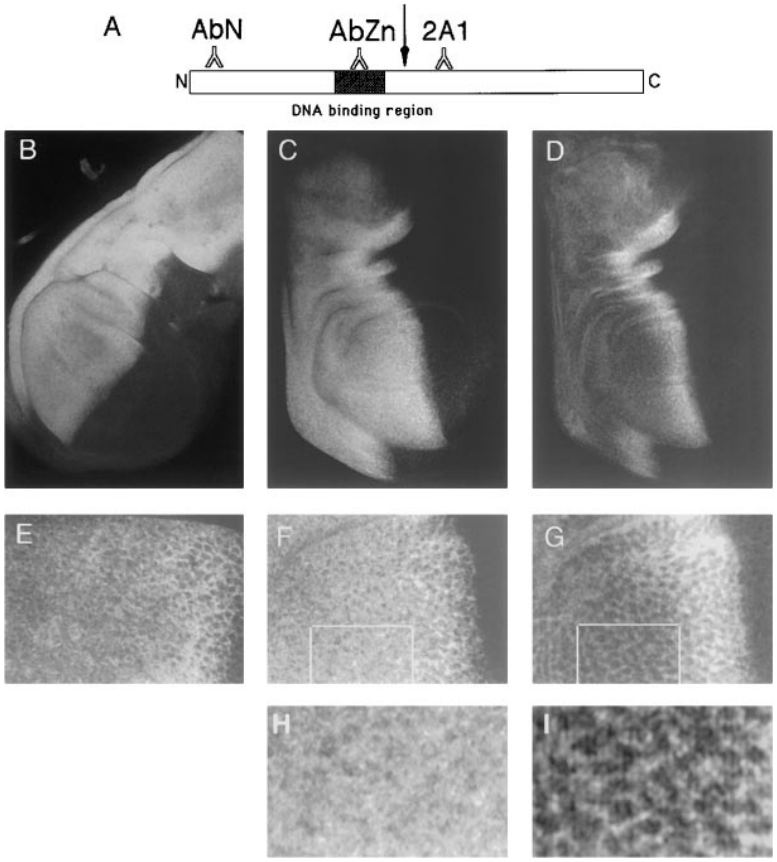

Figure 1. Varied Distributions of Ci Epitopes

(A) Map of the predicted Ci protein with its zinc finger domain; approximate location of epitopes recognized by AbN, AbZn and MAb 2A1; and approximate location of cleavage site.

(B–D) AbN (B) and AbZn (C) bind more uniformly than MAb 2A1 (D). Discs are oriented dorsal up and anterior left.

(E–G) Higher magnification views showing the regions of the A/P compartment borders stained with AbN (E), AbZn (F), and MAb 2A1 (G).

(H and I) High magnification views of boxed portions of (F) and (G) showing cytoplasmic staining of MAb 2A1 and uniform staining of AbZn.

The disc in (C), (F), and (H) and in (D), (G), and (I) has been stained with both AbZn and MAb 2A1.

are not. Cells near the A/P compartment borders have higher levels of Ci protein than do cells elsewhere in the A compartments (Motzny and Holmgren, 1995). The mechanism responsible for the contrasting distributions of *ci* RNA and protein is not understood, but it seems likely that these differences are related to the way in which cells near the compartment border respond to Hh protein. Conceivably, the Hh signal could increase the half-life of Ci protein, it could change the splicing pattern of the *ci* transcript, or it could influence post-translational processing.

To investigate how the nonuniform distribution of Ci protein is generated, we monitored Ci protein in wing imaginal discs with three antibody preparations (Figure 1). These were: AbN, AbZn, and MAb 2A1, which recognize the N-terminal, putative zinc finger, or C-terminal regions, respectively. Wing imaginal discs adsorbed AbN, AbZn, and MAb 2A1 exclusively to A compartment cells. Nevertheless, whereas MAb 2A1 prominently stained cells at the A/P compartment border and stained significantly less elsewhere in the A compartment (Figures 1D and 1G), AbN and AbZn staining was more uniform (Figures 1B, 1C, 1E, and 1F). To explore the possibility that different forms of the Ci protein are present in different parts of the A compartment, extracts of embryos, wing discs, and Cl-8, a tissue culture cell line derived from imaginal discs, were analyzed.

AbN recognized proteins with approximate molecular weights of 155 kDa and 75 kDa. Both proteins are produced in gastrulating embryos (Figure 2A), but neither is present in presyncytial blastoderm embryos (not shown). Both proteins are also produced by wing discs and Cl-8 cells. The size of the larger species is consistent with the predicted open reading frame of *ci* cDNAs (see Experimental Procedures; Orenic et al., 1990; Schwartz, et al., 1995) and with previous Western blot analyses (Slusarski et al., 1995). It is, presumably, the intact and full-length Ci protein, and it is recognized by all three antibody reagents. The 75 kDa species is a novel Ci product and is designated Ci75. It is recognized by both AbN and AbZn, but not by MAb 2A1 (Figure 2B). This suggests that the wing disc staining patterns of AbN, AbZn, and MAb 2A1 reflect different distributions of full-length Ci and of Ci75.

Ci75 Is Produced by Proteolysis

Tra-1 is a C. elegans gene whose sequence is closely related to *ci* and which generates two distinct mRNAs by alternative splicing (Zarkower and Hodgkin, 1992). Our observation that Drosophila has two forms of Ci protein raises the possibility of an analogous process in flies. Alternatively, the two forms of the Ci protein could be produced by posttranslational processing. To distinguish between these possibilities, we carried out a pulse–chase analysis of Ci in Cl-8 cells. After a pulse of ^{35}S-methionine, samples were collected at regular intervals, immunoprecipitated, and fractionated. As shown in Figure 2C, 84% of the signal was in full-length protein after the pulse labeling. During the subsequent chase, the total signal remained constant (30,111 at

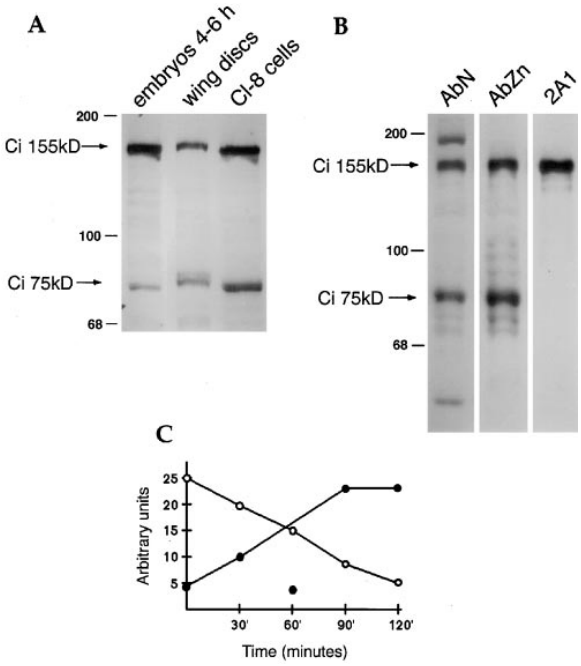

Figure 2. Ci Is Cleaved to Generate a 75 kDa Form

(A) Western blot analysis of Ci protein with AbN reveals both full-length Ci (155 kDa) and the 75 kDa form in embryos (lane 1), third instar wing discs (lane 2), and Cl-8 cells (lane 3).

(B) Western blot analysis of Cl-8 extracts AbN, AbZn, and Ab 2A1.

(C) Pulse–chase labeling of Cl-8 cells analyzed for full-length Ci (closed circles) and Ci75 (open circles). Measured values for Ci75, which has 1/4 of the Met residues as full-length Ci, have been multiplied by 4.

time = 0; 28,473 at time = 2 hr); however, the proportion of signal in full-length protein and Ci75 reversed. Most of the label (82%) was in Ci75 after two hours. We estimate that full-length Ci has a half-life of approximately 75 min in Cl-8 cells and conclude that Ci75 is generated from full-length Ci by cleavage. We estimate that the cleavage that generates Ci75 is between residues Leu-650 and Asp-700 (Figure 1A), and we confirmed that cells transfected with a construct that terminates translation at Asn-703 make a protein with slightly slower electrophoretic mobility than Ci75 (Figure 3A).

Ci75 Is a Nuclear Protein

Previous reports described Ci protein as predominantly or entirely cytoplasmic, despite its five putative zinc fingers and presumed role as a transcription factor (Orenic et al., 1990; Schwartz et al., 1995). We confirmed these observations by immunostaining embryos and imaginal discs with MAb 2A1 (Figures 1G and 1I). To determine whether the subcellular location of Ci75 is different from that of the full-length protein, we placed a construct encoding the C-terminal deletion lacking residues C-terminal to Asn-703 (Ci76) under UAS control and expressed it in clones of cells in transgenic flies. Ci76 in wing disc clones was almost entirely nuclear (Figure 7B). A similar result was obtained when Ci76 was expressed in S2 cells (Figure 7D). We conclude that the Ci protein sequence N-terminal to Asn-703, which contains the five putative zinc fingers, includes sequences that direct it to the nucleus. These results are consistent with the observations of Hepker et al. (1997), who also characterized C-terminal Ci deletions.

To confirm the subcellular location of Ci75, extracts of Cl-8 cells were separated into cytoplasmic and nuclear fractions by centrifugation. Western analysis revealed that full-length Ci protein was primarily in the cytoplasmic fraction and Ci75 was almost exclusively in the nuclear fraction (Figure 3B).

Ci76 Represses *hh* and *ptc*

To investigate the functional activity of C-terminally truncated Ci protein, the Ci76 transgene was expressed in various patterns. When expressed under *ptc* control, Ci76 caused lethality at 25°C. However, numerous escapers were recovered at 16°C, and these had abnormal wing phenotypes (Figure 4A) that are similar to ci^{Ce} and to some *fu* alleles. This observation suggests that expression of Ci76 along the A/P compartment border may block the response of the A cells to Hh protein. Staining wing discs with anti-Ptc antibody confirmed that *ptc* expression was reduced at the A/P compartment border (Figure 4C). Although the wings were malformed, their size was not abnormal, suggesting that *dpp* was expressed at levels sufficient to promote normal development. Staining wing discs with anti-Dpp antibody revealed an unusually broad band (Figure 4D), but its intensity was not obviously abnormal.

We also expressed Ci76 in the P compartment by placing it under *en* control (*en*-GAL4). *en*-GAL4 UAS-Ci76 animals were inviable at 25°C, but escapers with severe defects were obtained by raising animals at 25°C after an embryonic period at 16°C. These flies had defects that ranged to almost complete loss of wings, severely disrupted legs, and reduced thorax, and a wing representative of an intermediate malformation is shown in Figure 4B. These phenotypes recall the consequences of reduced *dpp* function, suggesting that ectopic expression of Ci76 in the P compartment reduced *dpp* expression. Since *dpp* is normally expressed only by A compartment cells that respond to Hh protein, it seems plausible that Ci76 suppressed *hh* expression in the P compartment in these discs. We therefore monitored the levels of Ci, Ptc, En, and Hh in discs expressing Ci76 in their P compartments. In the *en*-GAL4 system we used, ectopic expression was most pronounced in the wing pouch and less intense elsewhere in the wing or leg discs. Consistent with this, Ci76 was detected most prominently in the wing pouch. In contrast, full-length Ci, Ptc, and Dpp were present only in A cells at the compartment border of wing discs at some distance from regions with high levels of Ci76 (Figure 4). Levels of En protein were not affected in any of the discs. We attribute the Ci, Ptc, and Dpp patterns to the significant reduction of Hh in the P compartment of wing discs (Figure 4H). Note that the wing and haltere discs were abnormally small and that Hh levels were reduced in both discs (Figure 4H). In contrast, the leg disc was of normal size and had normal levels of Hh. This difference is consistent with the greater relative expression of *en*-GAL4 in the dorsal discs, but may also reflect a lower sensitivity to ectopic Ci76.

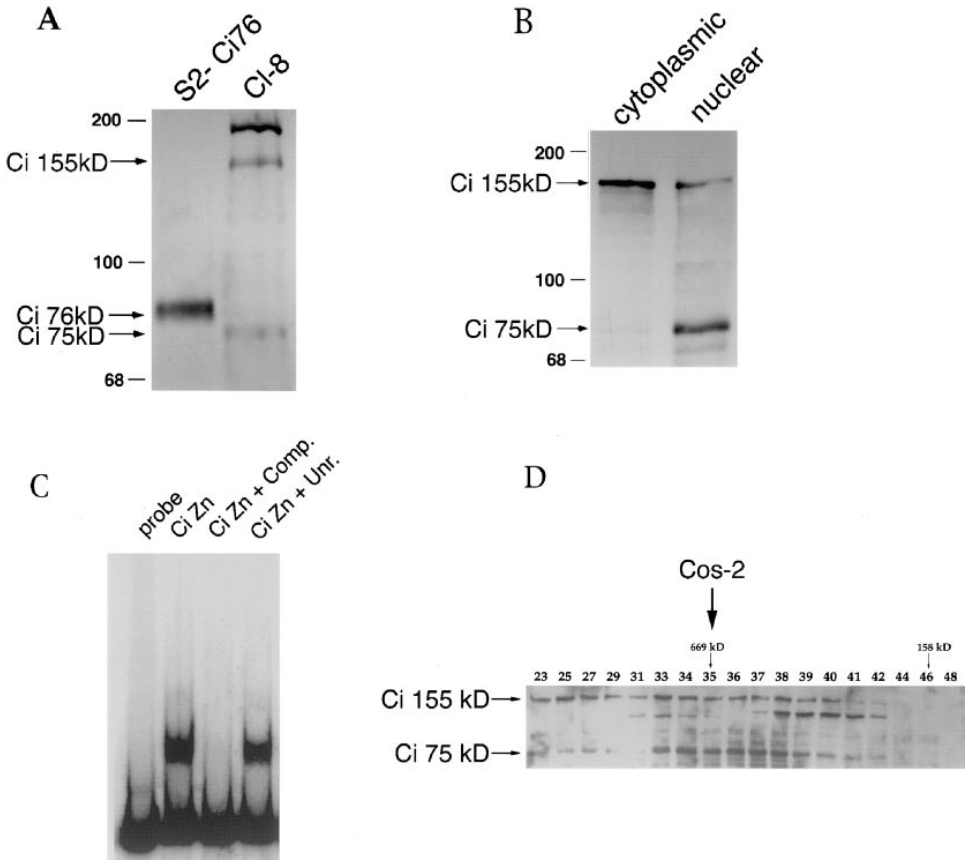

Figure 3. Ci75 Is a Nuclear DNA-Binding Protein

(A) Western blot analysis showing Ci76 expressed in S2 cells running at a slower mobility than Ci75 isolated from Cl-8 extracts.

(B) Cl-8 cells separated into cytoplasmic (lane 1) and nuclear (lane 2) fractions by differential centrifugation analyzed by Western blot analysis with AbZn.

(C) Gel retardation assays showing binding by purified His-CiZn (lane 2) to a DNA fragment from the *ptc* promoter containing GLI consensus binding sites. Binding was competed by DNA containing GLI sites but not by unrelated sequences.

(D) Western blot of fractions collected after gel filtration, showing that full-length Ci and Ci75 elute in broad peaks and that Cos-2 (arrow) cofractionates. Fractions in which MW markers thyroglobulin (669 kDa) and aldolase (155 kDa) eluted are indicated.

We conclude that ectopic expression of Ci76 in P cells severely reduces expression of *hh* and has secondary consequences on *ptc* and *dpp* expression in A cells across the compartment border. Ectopic expression of full-length Ci has quite different effects. Full-length Ci in the P compartment induces *ptc* expression (not shown; Alexandre et al., 1996; Domínguez et al., 1996) but has no effect on *hh* expression. These experiments therefore suggest that proteolysis of Ci can generate a protein with an activity distinct from that of the full-length protein. The observed nuclear localization of Ci76 and the repression of *ptc* and *hh* in cells that ectopically express Ci76 indicates that the N-terminal fragment of Ci is a repressor.

Ci Protein Has Sequence-Specific DNA-Binding Capacity

The capacity of Ci protein sequences to bind DNA was tested in two ways. First, the DNA-binding activity of a fragment of Ci containing the putative zinc finger region was fused to a His tag (His-CiZn), and gel retardation assays were performed in the presence of a fragment of the *ptc* promoter that contains several potential GLI consensus binding sites. His-CiZn binds efficiently and specifically to the DNA fragments containing these sites (Figure 3C).

Ci76 produced in transfected S2 cells also binds GLI sites; however, most of the DNA–Ci76 complex did not migrate a significant distance in the gel (not shown). This observation suggests that the size of this complex may be greater than DNA–His-CiZn. Purification of Ci and Ci75 from embryos and Cl-8 cells revealed that both proteins sediment in a sucrose gradient and elute from a gel filtration column with an apparent size much greater that 150 kDa. Ci and Ci75 elute from a gel filtration column with an apparent MW in excess of 5×10^5 and coelute with two other segment polarity gene products, Cos-2 (Figure 3D) and Fu (data not shown). These results are consistent with the observations of Robbins et al. (1997) and suggest that the size of the DNA–Ci76 complexes might reflect an association of Ci and Ci75 with Cos-2, Fu, and possibly other proteins.

The second approach tested DNA binding in vivo with a Ci construct that fused GFP to the N terminus (GFP-Ci). Expression of GFP-Ci in the salivary glands of transgenic larvae revealed specific bands of fluorescence (Figure

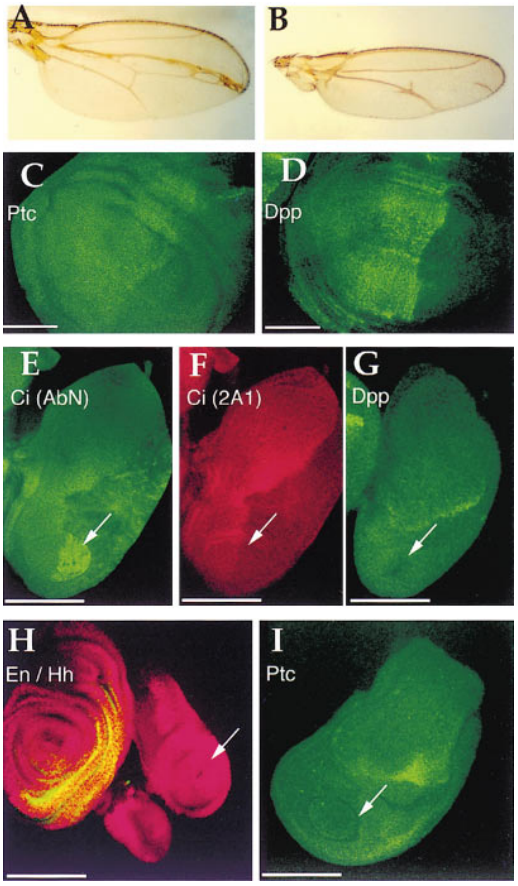

Figure 4. Ci76 Represses Ci-Target Genes In Vivo

Flies with *ptc*-GAL4 (A, C, and D) or *en*-GAL4 (B, E–I) and UAS-Ci76 were examined for wing phenotype (A and B), or for Dpp protein (D and G), Ptc protein (C and I), Hh protein ([H], green), En protein ([H], red), or, Ci protein with AbN (E) or MAb 2A1 (F) in third instar imaginal discs. Arrows point to wing pouch; all discs are wing discs except in (H), which also includes a leg and haltere disc. Wing and haltere discs in (H) are examples of more extreme effects on *hh* expression than (E, F, G, and I). Scale bars represent 250 μm.

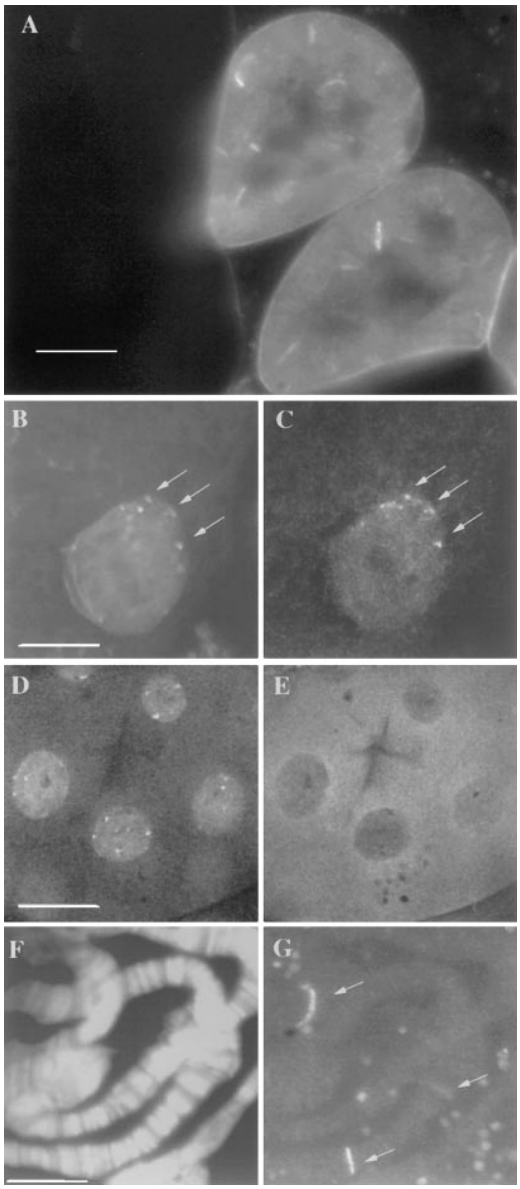

Figure 5. Ci75 Binds to Polytene Chromosome Bands

Unfixed salivary gland nuclei (A) containing GFP-Ci have several sites of intense fluorescence. A fixed salivary gland nucleus containing GFP-Ci stained with AbN and viewed to reveal sites of GFP fluorescence (B) and bound AbN (C); arrows indicate bands that contain both anti-Ci and GFP. A similar preparation shows that GFP fluorescence (D) is nuclear, but MAb 2A1 binding (E) is mostly cytoplasmic. Spread chromosomes counterstained with Hoechst (F) have GFP fluorescence (G) at bands 44D, 48A, and 50A (arrows, top to bottom). Scale bars: 25 μm (A and B), 50 μm (D), and 10 μm (F).

5). Approximately 8–10 bright fluorescent bands (including cytological locations 3, 9A, 28A, 35B, 44D, and 50A) and 25 bands with less intense fluorescence were detected. Noteworthy is a band of less intense fluorescence at 48A, the cytological location of *en* and bright fluorescent bands at the regions in which the genes *ptc* (44D), *wg* (28A), and *Cos* (50A) are located (Figures 5F and 5G). Since sites of GFP fluorescence and AbN antibody binding coincide in these nuclei (Figures 5B and 5C), we conclude that these fluorescent bands represent sites of Ci binding.

However, full-length Ci is largely or entirely cytoplasmic (Figure 3B), so we probed the structure of the GFP-Ci protein produced in salivary glands. Adsorption of AbN and MAb 2A1 revealed that AbN binding was largely nuclear (Figure 5D), contrasting with the localization of the epitope recognized by MAb 2A1, which was predominantly cytoplasmic (Figure 5E). This suggests that the form of GFP-Ci that localized to the polytene bands had released its C-terminal domain, and Western blot analysis confirmed that these salivary glands contained both full-length and cleaved forms of GFP-Ci (not shown). These results are therefore consistent with our thesis that the N-terminal domain of Ci has nuclear localization and DNA-binding activities that are impotent in the full-length protein.

Proteolysis of Ci Protein Is Regulated by Hh

In contrast to CI-8 cells, S2 cells do not express *ci* and do not normally contain Ci protein (Figure 6A). To investigate whether Ci protein synthesized in S2 cells could be cleaved to produce Ci75, we generated a *ci*

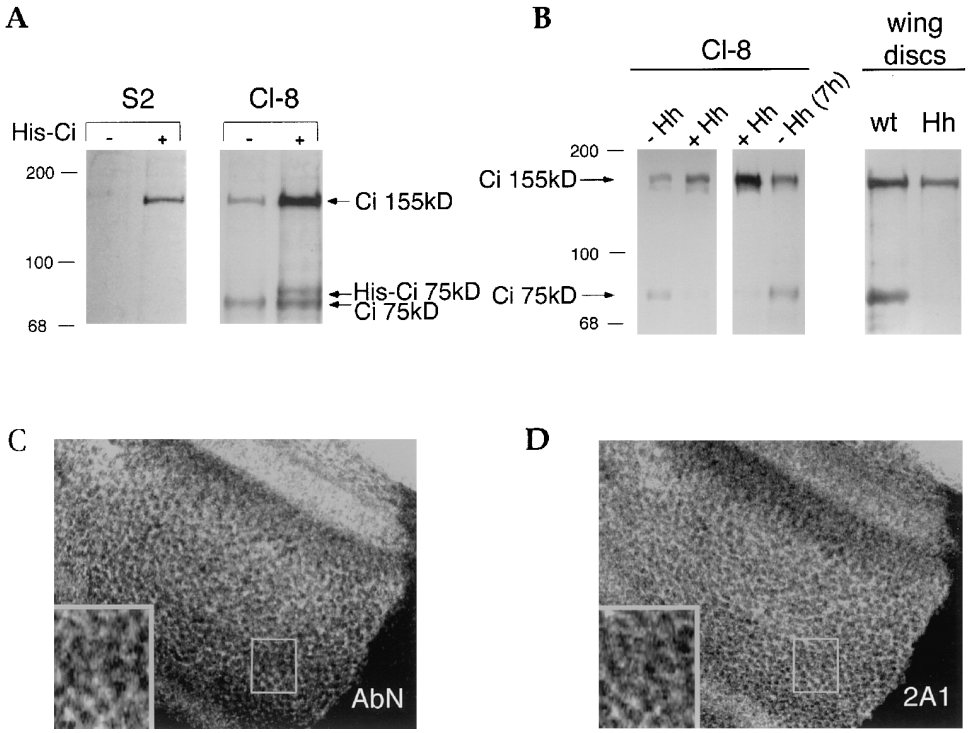

Figure 6. Proteolysis of Ci Is Regulated

(A and B) Western blots of S2 cells, Cl-8 cells, and wing discs probed with AbN; S2 cells ([A], left panel) and Cl-8 cells ([A], right panel; [B]) transfected with either GFP cDNA (−) or His-Ci75 (+). Only Cl-8 cells express *ci* endogenously and produce significant amounts of Ci75. ([B], left panel): comparison of Cl-8 extracts from untreated cells (−Hh) or cells treated with HhN-conditioned medium for 36 hr (+Hh). ([B], middle panel): extracts from cells incubated under the same conditions (+Hh) or returned to normal medium for 7 hr (−Hh). ([B], right panel): extracts of wild-type wing discs (wt) and mutant discs expressing a Hh-N transgene under *en* control (Hh). Twenty wild-type and twelve mutant discs were used, and Western was overexposed to increase the signal from the mutant discs; signal from wild-type is not representative of the relative intensity of the Ci and Ci75.

(C and D) Wing disc expressing N-Hh driven by an *en*-GAL4 strain is grossly enlarged and misshapen and shows little nuclear staining with either AbN (C) or MAb 2A1 (D). Higher magnification views of boxed regions show similar staining patterns with the two antibodies. P compartment is to lower right.

cDNA that had been mutated to encode 20 additional residues that include $(His)_6$ at the N terminus (His-Ci). This modification permitted His-Ci75 to be distinguished from Ci75, and after transfection into Cl-8 cells, Western analysis detected a novel cross-reacting band that migrated slightly slower than Ci75 (Figure 6A). In contrast, transfection of this construct into S2 cells generated only a full-length product. This result suggests that cleavage to generate Ci75 is neither intrinsic to Ci, nor is it an activity that is present in all cells.

If the relevant proteolysis is a specific and regulated function, then it may be controlled by Hh, and a model to account for the Hh-dependent Ci response can be formulated. Since Ci75 has a repressor activity that is distinct from the full-length protein, it is possible that cells receiving a Hh signal induce expression of target genes by inhibiting the formation of Ci75. Cells that receive the Hh signal accumulate elevated levels of cytoplasmic Ci, and the distribution of Ci epitopes changes with distance from the A/P compartment border and source of Hh protein. In addition, MAb 2A1 staining was always cytoplasmic (Figures 1G and 1I), but AbN and AbZn staining was in both the nuclear and cytoplasmic compartments (Figures 1E, 1F, and 1H), except at the A/P border, where it appeared to be mostly cytoplasmic. Since this position-dependent distribution of the AbN and AbZn epitopes correlates with cells responding to Hh, we tested the dependence of Ci proteolysis on Hh.

To monitor the response of Ci to *hh* signaling, Cl-8 cells were maintained in the presence of Hh for up to 36 hr prior to Western analysis. Full-length Ci protein accumulated in cells treated with Hh, while the level of Ci75 diminished (Figure 6B). Elevated levels of full-length Ci could be detected as early as 3 hr after addition of Hh (not shown). Since levels of *ci* mRNA did not change after addition of Hh (D. Casso and T. B. K., unpublished data), it seems that the half-life of full-length Ci protein is lengthened by Hh signaling, and we suggest that this difference is a consequence of reduced levels of conversion to Ci75. The Hh-dependent inhibition of Ci proteolysis is reversible. If cells that had been maintained in the presence of Hh were washed extensively and recultured in the absence of Hh, elevated levels of Ci75 could be detected within 4 hr (not shown) and levels returned to normal within 7 hr (Figure 6B).

We then investigated the action of Hh on generation of Ci75 in vivo. To test the prediction that elevated levels of Hh synthesis by P cells should suppress Ci75 production in a greater number of A cells, a gene construct encoding the diffusible form of Hh (Hh-N) was expressed

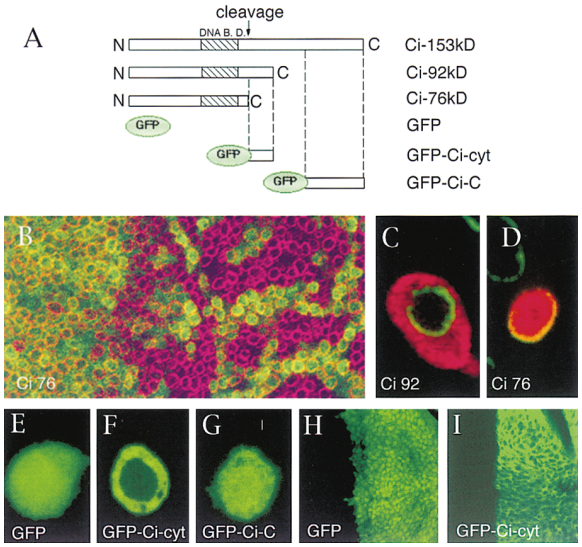

Figure 7. Localization Domains in Ci

(A) Diagram of constructs used to localize the cytoplasm-tethering domain.

(B) Third instar wing disc obtained after heat shock of larvae carrying UAS-Ci76, Ub>y^{+}>GAL4, and Hsp70-flp and stained with AbN (green) and antisera directed against the nuclear pore complex (red) reveals nuclear localization of Ci76. This view includes the region of the A/P compartment border; orientation is anterior left and dorsal up.

(C and D) S2 cells transfected with constructs to produce Ci92 (C) or Ci76 (D) stained with AbN (red) and anti–nuclear pore complex antibody (green).

(E–G) GFP fluorescence in S2 cells expressing GFP (E), GFP-CiCyt (F), or GFP-CiC (G).

(H and I) GFP fluorescence in third instar wing imaginal discs carrying *en*-GAL4 and UAS-GFP (H) or *en*-GAL4 and UAS-GFP-CiCyt (I). Anterior is left and dorsal is up.

under *en* control in P cells. In contrast to normal wing discs, discs expressing Hh-N stained relatively uniformly across the A compartment staining with both AbN and MAb 2A1, and staining was predominantly cytoplasmic (Figures 6C and 6D). Western analysis confirmed that the level of Ci75 was severely reduced in these discs (Figure 6B). We conclude that the relative amounts of full-length Ci and Ci75 depend upon the Hh signal, and suggest that modulation of *ci* processing may be an important part of response to the *hh* signals.

Ci Is Tethered in the Cytoplasm through a C-Terminal Domain

The differential localization of full-length Ci and Ci75 suggests that either the nuclear localization signals are masked in full-length Ci or that Ci is actively sequestered in the cytoplasm. To address the mechanism of selective localization, a set of deletions that remove increasing numbers of nucleotides from the 3′ end of the Ci coding region were generated (Figure 7A), and after transfection into S2 cells, the subcellular distribution of the Ci protein derivatives was monitored by antibody staining. Relevant constructs are Ci92 (deleted after Met-850), whose localization is indistinguishable from that of full-length Ci (Figure 7C), and Ci76, which is deleted after Asn-703 and is a nuclear protein (Figure 7D). This suggests that residues between 703 and 850 tether Ci in the cytoplasm, and to test this prediction directly, a fusion of GFP and residues 703–850 was made (GFP-CiCyt). S2 and CI-8 cells transfected with the construct encoding GFP-CiCyt had bright cytoplasmic fluorescence but little fluorescence in nuclei (Figure 7F). In contrast, cells transfected with GFP alone had fluorescence throughout and most prominently in nuclei (Figure 7E). As an additional control, a fusion between GFP and the C-terminal 330 residues of Ci was generated (GFP-CiC). This fusion protein does not include the Ci residues in GFP-CiCyt, and its distribution in transfected cells (Figure 7G) was indistinguishable from that of GFP alone. This confirms that the distribution of CiCyt is a function of the domain in residues 703–850 of Ci.

To verify the activity of CiCyt in cytoplasmic tethering, the distribution of GFP fluorescence was monitored in imaginal discs expressing GFP-CiCyt. Cytoplasmic localization of GFP fluorescence was detected after GFP-CiCyt was expressed under control of *en*-GAL4 (Figure 7I) or of *vg*-GAL4 (not shown). Similar preparations with UAS-GFP alone had fluorescence throughout the imaginal disc cells (Figure 7H). No differences in localization of GFP-CiCyt were evident at or with respect to the A/P compartment border. Although is is possible that a small fraction of the GFP-CiCyt translocates to the nucleus in response to Hh, our data are consistent with the proposal that Hh has no direct effect on the sequestration of the CiCyt domain itself.

Discussion

Ci plays a dual role in the A compartment where it represses *hh* to allow for anterior development (Domínguez et al., 1996) and functions as a component of the Hh signal transduction pathway at the A/P compartment border. Although previous studies based models of Ci function on the apparent increase in Ci concentration in A cells at the compartment border (Domínguez et al., 1996; Hepker et al., 1997), our data indicate that the actual levels increase only slightly (Figure 1). We found instead that processing of Ci protein can change the composition and distribution of Ci in A cells, thereby generating different forms of Ci that react differentially with anti-Ci antibodies. Since Ci proteolysis is inhibited by Hh, we suggest that it is by regulating the form of Ci, not its absolute amount, that the differential activity of Ci is controlled.

Ci Is a Transcription Factor

Sequence comparisons and analysis of in vivo activity have indicated that Ci has activities homologous to the GLI transcription factors (Orenic et al., 1990). We now show that Ci can recognize specific sites on Drosophila chromosomes. A GFP-Ci fusion protein produced in larval salivary gland cells places GFP fluorescence at specific polytene bands (Figure 5). Some of these bands correspond to regions in which Hh target genes are thought to be located. Although we have not yet shown that these presumptive target genes bind GFP-Ci directly, available sequence information confirms that GLI consensus binding sites are present in at least three: *ptc*, *wg*, and yolk protein 3. In the absence of additional

sequence information, we do not know if GLI sites are present at the other locations, but our observations are consistent with the proposal that the sites bound by GFP-Ci include the putative target genes.

Our data indicate that the active nuclear form of Ci protein is not full-length, but is a proteolytic product generated from the full-length protein. MAb 2A1, which recognizes an epitope C-terminal to the zinc finger region, does not recognize GFP-Ci in polytene nuclei (Figure 5E), suggesting that GFP-Ci that localizes in these nuclei lacks its C terminus. Although it is possible that the MAb 2A1 epitope is masked in the nuclear form of GFP-Ci, or that alternative splicing generated a shortened form that lacks the C terminus, our evidence for cleavage of Ci in the Cl-8 cells makes these alternatives less likely. Proteolysis generates Ci75 from Ci and also generates a form of GFP-Ci in salivary glands that binds specifically to a limited number of chromosomal sites. We do not yet know if Ci75 is the only active form of Ci, if the full length can translocate to the nucleus and engage the transcriptional apparatus under some conditions, or if different processing pathways generate alternate active forms. We favor the latter, especially in view of the recent finding that CBP interacts with C-terminal sequences of Ci and that this interaction is required to activate target genes (Akimaru et al., 1997).

Ci75 Is a Repressor Form of Ci Whose Production Is Inhibited by Hh

Ci75 is produced by anterior cells where we propose that it functions to repress target genes. Several observations support this proposal. First, Ci75 is a nuclear protein. It can be isolated with the nuclear fraction (Figure 3B), and wing imaginal disc nuclei stain with AbN, which recognizes both Ci75 and full-length Ci, but not with MAb 2A1, which recognizes only full-length Ci. Since AbN recognizes both full-length Ci and Ci75, these observations suggest that full-length Ci is predominantly cytoplasmic and that the nuclear staining represents Ci75. Second, the presence of Ci75 correlates with repression of *hh* and *ptc* expression. A cells that do not normally express *hh* do so if they lose *ci* function (Domínguez et al., 1996), and P cells, which normally express *hh*, cannot if they express Ci76 (Figure 4H). Regulation of *ptc* expression is more complex, although there appears to be a direct relationship between the presence of Ci75 in A cells removed from the A/P compartment border and low level of *ptc* expression and between the repression of *ptc* expression in A cells at the border when they ectopically express Ci76.

We also propose that Hh inhibits the proteolytic reaction that produces Ci75. This proposal is based on the observation that AbN stains nuclei of A cells at the compartment border at a diminished level (Figure 1E), that Ci75 disappears from Cl-8 cells treated with Hh-conditioned medium (Figure 6B), and that in wing discs overexpressing Hh, levels of Ci75 and of nuclear staining decrease (Figures 6B and 6C). It is interesting to speculate on the consequences of this regulatory circuit on *hh* and *ptc* expression.

If A cells at the compartment border receiving the Hh signal reduce production of Ci75, then the question of why these cells do not express *hh* arises. We cannot provide an explanation other than to offer that either sufficient levels of Ci75 remain in these cells to repress *hh*, or that *hh* is repressed by another, as yet unknown, factor in these border cells. This dilemma may not be relevant to the eye imaginal disc, where the border between *ci*-expressing cells and *hh*-expressing cells is not static. In eye imaginal discs, a wave of differentiation progresses across the disc from the P margin and is marked at its forward edge by a morphogenetic furrow. The morphogenetic furrow employs the Hh and Dpp signaling pathways to organize ommatidial clusters, with Hh inducing *dpp* expression in more anterior cells in the furrow (reviewed by Heberlein and Moses, 1995). The morphogenetic furrow can be considered in this context to represent a moving compartment border, although it is not yet understood how the movement of the furrow is propelled. It seems conceivable that the action of Hh inhibiting production of Ci75 in cells on the leading edge of the morphogenetic furrow in the eye disc might enable these cells to express *hh* and then to signal their anterior neighbors in a like manner. If this process is sequential and repetitive, it would propel furrow movement.

With regard to *ptc* regulation, we presume that in that absence of the Hh signal, Ptc actively promotes formation of Ci75, since *ptc* mutant clones accumulate full-length Ci protein and by inference do not proteolyze Ci. This is in accord with previous proposals that Ptc has a constitutive activity in the absence of Hh and would suggest that the effect of Hh is, at least in part, to suppress this positive effect of Ptc on Ci75 production. Viewed in this way, Hh negative regulation of Ptc and Ptc negative regulation of *ptc* expression (Ingham et al., 1991) can be understood through the regulated action of Ci75.

ptc is induced in P cells that ectopically express full-length Ci (this work; Alexandre et al., 1996; Domínguez et al., 1996), but it is not induced in *ci* mutant clones (Domínguez et al., 1996), suggesting that either Ci75 does not repress *ptc* or that expression of *ptc* requires a distinct Ci activator function. We favor the latter explanation. Since ectopic expression of full-length Ci in the P compartment induces *ptc* (this work, data not shown; Alexandre et al., 1996), but ectopic activation of Ci75 does not (Figure 4I), an activator function appears to be associated with expression of full-length Ci. Indeed, Alexandre et al. (1996) suggested that the C-terminal region of Ci might contain a transcriptional activation domain, and N-terminally truncated Ci protein can activate *ptc* expression (Hepker et al., 1997). These observations open the possibility that alternative processing pathways might convert Ci to either an activator or repressor form. The simplest model is that *ptc* is directly induced by an activated form of the full-length Ci protein, although it is possible that proteolysis of the full-length protein is involved in generating a Ci activator. However, if this basic idea is correct, then *ptc* expression may sense a balance between repressor and activator forms of Ci. This model is in accord with the increase in *ptc* expression in A cells at the compartment border, because formation of Ci75 is inhibited and the putative Ci activator would be expected to predominate. We would therefore propose that the various regulatory targets of

Ci are designed to respond to different ratios of activator and repressor.

Our observations are not directly relevant to *dpp* regulation, as we did not observe a direct correlation between either Ci75 or full-length Ci and *dpp* expression. Although we can only propose that Ci control of *dpp* is indirect, it is relevant to mention that expression of a transgene encoding the Ci zinc finger domain coupled to the En repression domain elicits an effect on *dpp* expression similar to Ci76 (Hepker et al., 1997). This observation is consistent with our suggestion that Ci76 is a repressor.

Subcellular Localization

A domain C-terminal to the zinc finger region (CiCyt) sequesters Ci in the cytoplasm (Figure 7), and Ci75, which lacks CiCyt, is primarily nuclear. Our data do not address whether nuclear transport of Ci76 is regulated even in the absence of CiCyt, and it is possible that additional levels of regulation may control the activity of Ci and its derivatives. Nevertheless, sequestration of Ci in the cytoplasm infers that CiCyt directly interacts with structural components in the cytoplasmic compartment. Since Ci forms a stable multiprotein complex that includes Fu and Cos-2 (see Results; Robbins et al., 1997; Sisson, et al., 1997), these proteins are obvious candidates.

Regulating Ci Activity

There are several transcription factors whose activity is in part regulated by nuclear transport, and a number of these are activated after limited proteolysis (see, for instance, Fan and Maniatis, 1991; Wang et al., 1994). We suspect that both the susceptibility of Ci to proteolysis and the activity of the responsible protease are regulated. For example, production of Ci75 was not observed in S2 cells, even though these cells can respond to Hh by phosphorylating their Fu protein (Thérond et al., 1996). S2 cells cannot process Ci protein efficiently, either because they do not express or have reduced levels of the protease, or are not able to activate it. Apparently, they are not fully equipped to respond to Hh. A likely candidate controlling the susceptibility of Ci to proteolysis is PKA, since PKA antagonizes the Hh signal response (Jiang and Struhl, 1995; Lepage et al., 1995; Li et al., 1995; Pan and Rubin, 1995). Ci has several potential sites for PKA phosphorylation, and levels of Ci protein appear to be elevated in clones of cells that lack PKA function (Johnson et al., 1995), an indication of low rates of proteolysis and Ci75 production.

Lastly, it is interesting to consider the possibility that regulated cleavage and activation of Drosophila Ci mirrors similar processing of the vertebrate Gli homologs. Although we know little of how the family of human GLI proteins is regulated, it appears that controlling their activity is critically important, since up-regulation of GLI is associated with gliomas and other forms of cancer (Kinzler et al., 1987) and down-regulation of GLI3 is associated with severe birth defects (Kang et al., 1997). Proteolysis of the Gli proteins may be essential to their proper regulation. It is also possible that posttranslational processing of the Drosophila protein generates homologs of the different vertebrate Gli proteins, and it should be possible to distinguish between these different alternatives.

Experimental Procedures

Ci cDNA and Expression Constructs

Corrections to the published and amended *ci* cDNA sequence have been submitted to GenBank. All constructs were in the pUAST vector (Brand and Perrimon, 1993), and numbering is such that the open reading frame starts at nucleotide 416. UAS-Ci76: truncation of *ci* cDNA extending to Asn-703 with a deletion from 2771 to 2974. UAS-Ci92: truncation extending to Met-850 due to an internal deletion from 2965 to 3965, with a 13 residue tail. UAS-His-Ci: construct produces full-length Ci with a 20 residue peptide including a 6× His tag (from pET15b [Novagen]) at the N terminus. UAS-GFP-Ci-cyt: residues 675–860 fused on the C-terminal side of GFP65T. UAS-GFP-CiC: residues 1066–1396 fused at the C-terminal side of GFP65T. His-CiZn: residues 1740–2278 of the *ci* cDNA subcloned into pET15b, expressed in *E.coli*, and purified with TALON Metal Affinity Resin (Clontech) in the presence of 8M urea, and renatured by dialysis into Tris (pH 8), 260 mM NaCl.

Pulse–Chase Experiment

Cl-8 cells were cultured in 10 cm dishes, at 25×10^6 cells per dish (24 hr) and washed three times with Met^-, Leu^- M3 medium. Cells were labeled with 4 ml/dish Met^- medium with 400 μCi of Trans ^{35}S Label (ICN) (45 min), washed three times with Met^-, Leu^- medium, and incubated in standard M3. Lysis was in 800 μl/dish of RIPA buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS) with protease inhibitors. RIPA efficiently extracts full-length Ci and Ci75. Extracts were precleared for 30 min with 40 μl of protein A–Sepharose that had been prepared 1:1 vol. in RIPA buffer, 2% BSA and incubated with 4 μl affinity-purified AbZn (1 hr). Immune complexes were precipitated with 40 μl of protein A-Sepharose (1 hr), washed (three times, 10 min, RIPA buffer), and extracted with 30 μl of 1× loading buffer, 20 μl of which was fractionated and quantified with a STORM phosphorimager, yielding values scaled in arbitrary units.

Western Blotting

Samples were obtained as follows: in Figure 2A, embryos and Cl-8 cells were extracted in RIPA buffer in the presence of protease inhibitors, and imaginal discs were lysed directly in gel loading buffer. Lanes were loaded with: lane 1, 34 μg of protein; lane 2, equivalent of 20 late third instar wing imaginal discs; and lane 3, 25 μg of protein. Samples in Figures 2B, 3B, 6A, and 6B were made by lysing cells in 20 mM Tris–HCl (pH 8.0), 100 mM NaCl, 8M urea and loading equal amounts of protein.

Gel-Retardation Assay

Binding conditions used were as described for GLI (Kinzler and Vogelstein, 1990). Probe was synthesized in a PCR reaction to amplify the Drosophila genomic DNA region between positions −1618 and −1746 of the *ptc* promoter. Loading was 2 ng of DNA per lane, with 10 ng His-CiZn, and where indicated, 200 ng of nonlabeled probe as competitor, or 200 ng of unrelated sequence.

Nuclear and Cytoplasmic Extracts Preparation

Cl-8 cells in semiconfluent 10 cm^2 dishes were washed in PBS, resuspended in 1 ml of 20 mM HEPES (pH 7.9), 10 mM KCl, and incubated on ice for 15 min. Nuclei and cellular debris were separated by centrifugation, and the pellet was resuspended in 2 ml of 20 mM HEPES (pH 7.9), 10 mM KCl, 350 mM sucrose (HKS), and resuspended with a B pestle dounce. The homogenate was loaded on 4 ml of HK + 2M sucrose, and free nuclei were separated from cellular debris and unbroken cells by centrifugation. The nuclear pellet was resuspended in 2 ml of HKS, pelleted by centrifugation, resuspended in HK, and pelleted. The nuclear pellet was resuspended in 100 μl of HK + 0.4 M NaCl and incubated on ice for 20 min, and the soluble nuclear fraction was collected after centrifugation. Each lane was loaded with 6 μg of protein.

Nondenaturing solutions used for protein extractions included: 10 μM Benzamidine HCl, 1 μg/ml Phenantroline, 10 μg/ml Aprotinin, 10 μg/ml Leupeptin, 10 μg/ml Pepstatin A, and 100 μM PMSF.

Cell Culture and Transfections

Conditioned medium containing Hh-N was obtained as described (Thérond et al., 1996). Cl-8 cells were cultured as described (van Leeuwen et al., 1994). Transfections were by calcium-phosphate; analyses were carried out 24–36 hr later. UAS constructs were co-transfected with 1/10 of the amount of Ub-GAL4.

Ectopic Expression of Ci

Ci76: early third instar larvae with a P element insert containing Ub>y+>GAL4, Hsp70-flp, and UAS-Ci76 were heat shocked for 30 min at 37°C and analyzed 24 hr later. Expression in *ptc* or *en* patterns was with *en*-GAL4 or *ptc*-GAL4. For each experiment, one high and one low expressing UAS-Ci76 strain was analyzed.

GFP-Ci: the construct was a fusion of GFP65T (Rizzuto et al., 1996) at the N terminus and a Myc tag at the C terminus of Ci, placed downstream of a UAS promoter. Expression was in salivary glands of third instar *ptc*-GAL4 larvae. Salivary glands were dissected in PBS, 0.5% Triton X-100; transferred to a drop of PBS, 1% Brij-58; and incubated for 1 min. An equal volume of 4 M urea was added, and after 30 sec to 1 min, glands were rinsed four times with PBS, 1 μg/ml Hoescht 33258 and squashed.

Immunohistochemistry

Standard protocols were used for immunostaining discs (Tabata and Kornberg, 1994) and cultured cells (Harlow and Lane, 1988). Antibodies were: mouse anti-Nuclear Pore (Babco), rat monoclonal anti-Ci 2A1, rabbit anti-Ci (AbN) (Tabata et al., 1995), rabbit anti-Hh (Tabata and Kornberg, 1994), rabbit anti-Dpp, and mouse monoclonal anti-En 4D9 (Patel et al., 1989). Rabbit anti-Ci (AbZn) was raised against bacterially expressed His-CiZn. Western blotting with extracts of Schneider S2 cells transfected with various deletion constructs localized the epitope recognized by MAb 2A1 to the C-terminal side of the zinc finger domain, between Asp-700 and Met-850 (data not shown). Secondary reagents were from Jackson Immunologicals. Images were taken on a Laser Scanning Confocal Microscope.

Gel Filtration Assay

1.5×10^8 Cl-8 cells were collected and washed, resuspended in 2 ml of 50 mM Tris (pH 8), 50 mM NaF, 1 mM EDTA, 1% NP-40, 1/2 μl of PMSF, and 20 μl of protease inhibitor cocktail. After 10 min on ice, cells were homogenized with a B Dounce, and 0.26 ml of 4M $(NH_2)SO_4$ was added. The lysate was centrifuged for 30 min at 290,000 × g, and the resulting supernatant was applied to a Superose 6B FPLC column.

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