Dual pathways for induction of *wingless* expression by protein kinase A and Hedgehog in *Drosophila* embryos

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The secreted *Drosophila* Hedgehog (Hh) protein induces transcription of specific genes by an unknown mechanism that requires the serpentine transmembrane protein Smoothed (Smo) and the transcription factor Cubitus interruptus (Ci). Protein kinase A (PKA) has been implicated in the mechanism of Hh signal transduction because it acts to repress Hh target genes in imaginal disc cells that express Ci. Changes in Ci protein levels, detected by an antibody that recognizes an epitope in the carboxy-terminal half of Ci, have been suggested to mediate the positive effects of Hh and the negative effects of PKA on Hh target gene expression in imaginal discs. Here we show that PKA inhibition, like Hh, leads to increased “carboxy-terminal” Ci staining and Hh target gene expression in embryos. In addition, we find that Hh and Smo can stimulate target gene expression at constant Ci levels and that increased PKA activity can induce ectopic Hh target gene expression in a manner that requires Smo and Ci activities but does not involve changes in Ci protein concentration. This suggests a branching pathway of Hh signal transduction downstream of Smo and that PKA exerts opposite effects on the two branches. Finally we show that Hh signaling in embryos does not depend on cAMP-dependent regulation of PKA activity.

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Secreted proteins of the Hedgehog family act as key inducing agents in both vertebrates and invertebrates, often influencing position-dependent cell fate over a large field of cells (Ingham 1995). In some cases, including the *Drosophila* ventral embryonic ectoderm and larval imaginal discs, Hedgehog (Hh) acts locally to induce the synthesis of other secreted molecules, of the Wnt or transforming growth factor- β (TGF- β) families, that relay the influence of Hh to more distant cells (Lawrence and Struhl 1996). In other cases, Hh may act directly over several cell diameters, inducing concentration-dependent responses (Heemskerk and DiNardo 1994; Roelink et al. 1995; Ericson et al. 1996; Struhl et al. 1997). The direct range of action of Hh is limited by post-translational modifications to Hh itself and by binding to the Hh-receptor, Patched (Ptc), the product of a Hh-inducible gene (Chen and Struhl 1996; Porter et al. 1996). Genetic and biochemical interactions among Hh, Ptc, and Smoothed (Smo) suggest that binding of Hh to Ptc leads to activation of Smo, a predicted seven-transmembrane domain protein presumed to interact with a G protein (Alcedo et al. 1996; Chen and Struhl 1996; Marigo et al. 1996; Stone et al. 1996; van den Heuvel and Ingham 1996). Although protein kinases such as Fused (Fu) and

protein kinase A (PKA) are genetically implicated in Hh signaling, there is no evidence at present that their activities are altered by Hh signaling (Prat et al. 1990; Forbes et al. 1993; Jiang and Struhl 1995; Kalderon 1995; Lepage et al. 1995; Li et al. 1995; Pan and Rubin 1995; Strutt et al. 1995). Hh can signal normally in imaginal disc cells if PKA is manipulated to be unresponsive to cAMP (Jiang and Struhl 1995; Li et al. 1995) or if animals lack activity of both Fu and another gene product, Suppressor of Fused (Prat 1992; Thron et al. 1996a).

The zinc finger protein, Cubitus interruptus (Ci) has DNA-binding activity that is essential to transduction of a Hh signal, suggesting that it acts as a key transcriptional activator (Forbes et al. 1993; Alexandre et al. 1996; Von Ohlen et al. 1997). Ci staining with a monoclonal antibody, 2A1, that recognizes an epitope in the carboxy-terminal half of Ci, increases in response to Hh, loss of Ptc activity, or loss of PKA activity in anterior imaginal disc cells, without any change in *ci* mRNA levels (Johnson et al. 1995; Slusarski et al. 1995; Domnguez et al. 1996; Aza-Blanc et al. 1997). It has been shown recently that increased 2A1 antibody staining due to Hh signaling corresponds to a change in Ci processing rather than total Ci protein concentration, as assumed previously (Aza-Blanc et al. 1997). Thus, full-length Ci (Ci-155) can be cleaved to an amino-terminal fragment (Ci-75) that is not recognized by the “carboxy-terminal” an-

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tibody 2A1, henceforth denoted as Ci-CT antibody (Aza-Blanc et al. 1997). Hh inhibits the cleavage of Ci-155 and consequently leads to increased Ci-CT antibody staining while total Ci protein concentration, assayed by an "amino-terminal" (Ci-NT) antibody remains constant (Aza-Blanc et al. 1997). Because increased Ci-CT antibody staining generally accompanies induction of Hh target genes, whether elicited by Hh, PKA inhibition, or overexpression of a *ci* cDNA, it is thought that Ci-CT antibody staining, corresponding to an increased concentration of full-length Ci protein, is a critical intermediate step in Hh target gene induction (Johnson et al. 1995; Slusarski et al. 1995; Alexandre et al. 1996; Domínguez et al. 1996; Hepker et al. 1997). We have investigated Hh signaling in *Drosophila* embryos and find that PKA inhibition and Hh signaling increase Ci protein concentration detected by Ci-CT antibody and induce target gene [*wingless* (*wg*) and *patched* (*ptc*)] expression, as observed previously in imaginal discs. We also demonstrate novel signaling activities by which both PKA hyperactivity and Hh stimulate *wg* and *ptc* expression by a mechanism that requires the activities of Smo and Ci but does not involve any detectable changes in Ci protein concentration.

Results

PKA inhibition induces Hh target genes in embryos

In *Drosophila* embryos Hh is made in segmentally repeated stripes of *engrailed* (*en*)-expressing cells and is required to maintain expression of the target genes *wg* and *ptc* in stripes of immediately adjacent cells. *wg* is expressed anterior to each *en* stripe, whereas *ptc* is expressed on either side of *en*, forming two stripes per segment (Hidalgo and Ingham 1990) (Fig. 1a,b). *ptc* expression may serve to restrict diffusion of Hh (Chen and Struhl 1996) and moderate Hh signaling, whereas localized expression of *wg* is crucial initially to maintain *en* and *hh* expression and subsequently for ectodermal patterning within each parasegment (Peifer and Bejsovic 1992). Ubiquitous Hh expression can induce transcription of *ptc* in all cells that do not express *en* and that consequently express *ci* (Eaton and Kornberg 1990), whereas *wg* expression can be induced by Hh in a subset of those cells (Ingham 1993). Because the PKA catalytic subunit DC0 is required for normal oogenesis and maternally supplied in sufficient quantities for embryogenesis (Lane and Kalderon 1993, 1994) we used a mutant regulatory subunit, *R**, expressed from a GAL4-responsive transgene (*UAS-R**) to reduce PKA activity in embryos (Li et al. 1995). *R** binds cAMP poorly and therefore maintains catalytic subunit as inactive holoenzyme (*R**₂DC0₂) at physiological cAMP concentrations. Expression of *R** ubiquitously in the ectoderm ("*ubi-R**" = *E22C-GAL4* + *UAS-R**) or solely in non-*en* cells (using *ptc-GAL4*) caused an anterior expansion of *wg* expression (Fig. 1c) and expression of *ptc* in all non-*en* cells (Fig. 1d), without any accompanying change in *en* or *hh* expression (not shown). The alterations in *wg*, *ptc*, and ventral cuticle patterns (Fig. 5a,c, below) because of

Figure 1. Increased or decreased PKA activity results in activation of Hh target genes *wg* and *ptc*. (a,c,e,g) Ventral view of stage 11–12 embryos hybridized with *wg* RNA probe. Anterior is to the left. (b,d,f,h) Lateral view of embryos hybridized with *ptc* RNA probe. Anterior is left and dorsal is up. (a,b) Wild-type embryos showing the normal one-cell wide stripes of *wg* RNA and two thin stripes of *ptc* RNA in each segment of the ectoderm (clearest at the outer edge of the embryo in b). (c,d) *ubi-R** embryos in which PKA is inhibited uniformly. *wg* RNA expression expands 1–4 cells toward the anterior beginning in late stage 11 embryos, and *ptc* RNA expression is expanded to all non-*en*-expressing cells. (e,f) *ubi-mC** embryos in which PKA activity is uniformly elevated. The expansions of *wg* RNA expression are more robust than for *ubi-R**, reaching a width of 6–8 cells and are visible by early stage 11. *ptc* RNA expression expands to all non-*en* cells. (g,h) *ubi-R*/mC** embryos expressing both PKA transgenes have almost normal expression of *wg* and *ptc*, demonstrating the opposing activities of the two transgenes.

PKA inhibition in *ubi-R** embryos resembled those induced by low-level ubiquitous expression of Hh but were less pronounced than those elicited by high levels of Hh or strong *ptc* mutations (Bejsovic and Wieschaus 1993; Ingham 1993; Tabata and Kornberg 1994). PKA inhibition restricted to *en*-expressing cells (using *en-GAL4*) had no effect on *wg* or *ptc* expression (not shown). Hence, inhibition of PKA can mimic the inductive effects of Hh in Hh-responsive cells of the embryo, just as in imaginal discs.

Ci protein, detected by monoclonal antibody 2A1, is expressed in all non-*en* cells but is elevated in response to Hh at the borders of each broad segmental stripe (Motzny and Holmgren 1995) (Fig. 2b,h) and was induced to even higher levels throughout its normal expression domain by PKA inhibition in *ubi-R** embryos (not shown). PKA inhibition in alternating segments of "*alt-R**" [= *paired* (*prd*)-*GAL4* + *UAS-R**] embryos produced a cell-autonomous increase in Ci-CT antibody staining (Fig. 2a,d) without affecting *ci* RNA levels (Fig. 2f) or

Figure 2. Decreased but not increased PKA activity increases Ci-CT antibody staining. (a) Antibody to β -galactosidase reveals the expression pattern of GAL4 attributable to the *prd-GAL4* transgene in *alt-lacZ* (= *prd-GAL4* + *UAS-lacZ*) embryos. (b–h) Embryos stained with monoclonal antibody 2A1, which detects an epitope in the carboxy-terminal half of the full-length Ci protein. (d) In *alt-R** (= *prd-GAL4* + *UAS-R**) embryos there is a large increase in Ci-CT antibody staining in roughly alternating segments corresponding to those cells that express the PKA inhibitor (as in a), but *ci* RNA (f) is uniformly expressed in all non-*en* cells, as in wild-type embryos. The extent of induction of Ci-CT staining by PKA inhibition is better seen in Fig. 3. Here (d), it is underestimated because Ci staining is saturated to allow direct comparison to other embryos. (e) In *alt-mC** (= *prd-GAL4* + *UAS-mC**) embryos PKA hyperactivity does not alter Ci-CT antibody staining. (b,c,g,h) The focal plane differs from d and e to highlight accentuated Ci staining at the border of each band in wild-type embryos (b) that is absent from *ubi-mC** embryos (c) (PKA hyperactivity) and also absent from embryos zygotically mutant for *smo* (*smo*^{11x43}) (g) or (*hh*^{ts2}) (h).

Ci-NT antibody staining (not shown). Thus, PKA inhibition appeared to have similar effects to Hh on Ci staining, presumably corresponding to an increased concentration of full-length Ci protein and could induce ectopic Hh target gene expression in the embryo. However, PKA inhibition induced higher levels of Ci-CT staining than Hh (Figs. 2, b,d, and h; and 3c) but was less potent than Hh at inducing *wg* and *ptc* expression in *ubi-R** embryos (Fig. 1c,d), and especially in *alt-R** embryos (Fig. 3a,b), where only a slight expansion of *ptc* expression is seen and *wg* expression appears normal. This discrepancy provided the first hint that PKA or Hh might have additional functions that do not affect Ci protein levels.

Hh and Hh pathway components can induce wg expression independent of Ci protein levels

To test for additional functions of Hh we introduced Hh pathway mutations into *alt-R** embryos, in an attempt to circumvent the need for Hh to alter Ci protein levels. In *alt-R** embryos the induction of high levels of Ci-CT antibody staining (brown, Fig. 3c) anterior to the odd-numbered En stripes (blue, Fig. 3c) by PKA inhibition was not affected by *hh* or *smo* mutations (Fig. 3f,i). These embryos were double-stained with En antibody to identify the *hh* and *smo* mutant embryos among their siblings and were deliberately understained to show that the levels of immunoreactive Ci were not affected by *hh* or *smo* mutations (cf. Figs. 3c and 3f and i). In these understained embryos normal levels of Ci anterior to even-numbered En stripes are barely visible. Although they did not alter the effects of PKA inhibition on Ci staining, *hh* and *smo* mutations each eliminated expres-

sion of *wg* in *alt-R** embryos, even in those cells with high levels of Ci-CT antibody staining (Fig. 3a,d,g). Hence, Hh and Smo each provides an essential contribution to *wg* expression without detectably altering Ci protein levels, indicating a bifurcating pathway of Hh signaling downstream of Smo (Fig. 6, below). This is particularly clear in *alt-R** embryos lacking Smo activity, in which adjacent *wg* stripes decay at identical rates despite a huge difference in Ci-CT antibody staining between adjacent segments (Fig. 3g,i).

Expression of *ptc* RNA in response to PKA inhibition in alternating segments was still observed in *hh* mutants (Fig. 3e), suggesting a greater dependence of *wg* expression than *ptc* expression on the pathway of Hh signaling that does not alter Ci levels (Fig. 6, A, open arrow, below). In contrast, *smo* mutations eliminated expression of *ptc* in response to PKA inhibition (Fig. 3h), suggesting that Smo has a significant basal activity in the absence of Hh (Fig. 6, A, solid arrow, below). Expression of *wg* and *ptc* was not affected in *alt-R** or *ubi-R** embryos by loss of one active *ci* allele but was eliminated by complete loss of Ci activity (not shown).

Increased PKA activity also induces Hh target genes in embryos

A constitutively active mouse PKA catalytic subunit transgene, *mC**, was shown previously to oppose the elevated expression of Ptc protein in response to Hh in imaginal discs (Li et al. 1995). In stark contrast, expression of *mC** in embryos ubiquitously (*ubi-mC**) or under the influence of *prd-GAL4*, but not *en-GAL4*, caused ectopic expression of *wg* and *ptc* (Fig. 1e,f) without alter-

Figure 3. Hh and Smo can affect *wg* and *ptc* expression without altering Ci protein levels. Stage 10–12 embryos were hybridized with *wg* RNA (a,d,g), *ptc* RNA (b,e,h), or double-stained with Ci-CT antibody (brown) and En antibody (blue) (c,f,i). In *alt-R** embryos *wg* expression is close to wild type (a), *ptc* RNA is slightly expanded (b), but Ci-CT antibody staining is very high in cells of alternating segments that express the PKA inhibitor (c) (as in Fig. 2d). These embryos were stained more lightly for Ci than those in Fig. 2 so that normal levels of Ci protein are barely visible. (d–f) *alt-R** embryos also mutant for *hh* (*hh^{ts2}* at 29°C) lose *wg* (d) and En (f) expression in all segments by stage 10 but the levels of Ci-CT antibody staining remain very high (f) and *ptc* RNA is still expressed in cells of alternating segments that express the PKA inhibitor (e). (g–i) *alt-R** embryos lacking maternal and zygotic *smo* gene activity (*smo^{1x43}*) lose *wg* RNA (g), *ptc* RNA (h), and En expression (i) in all segments by stage 10 without alteration of high Ci-CT antibody staining in alternating segments (i). *alt-R** embryos lacking only zygotic *smo* have a similar phenotype, in which *wg*, *ptc*, and En expression decay at the same rate in adjacent segments and are substantially lost by the end of stage 11. *wg* and *ptc* expression also decay equally in adjacent segments of *alt-R** embryos lacking *ci* activity [*Df(4)M62f* or *ci^{DR50}*; not shown].

ing *en* or *hh* expression (not shown). The anterior expansion of *wg* stripes was more robust in *ubi-mC** than *ubi-R** embryos but was also only apparent after stage 11 and associated with ventral cuticles resembling those due to low-level ectopic Hh, rather than strong *ptc* mutations (Fig. 5d, below).

In embryos expressing both *mC** and *R** (*ubi-mC*/R**) *wg* and *ptc* expression patterns were close to wild type (Fig. 1g,h), as was Ci-CT antibody staining (not shown). This confirmed the opposing actions of the two transgenes on PKA activity, as was also verified biochemically (Li et al. 1995). In contrast to all other documented manipulations of Hh pathway components, PKA hyperactivity in *alt-mC** embryos caused ectopic expression of *wg* and *ptc* (Fig. 4a,b) without any increase in the level of *ci* RNA (not shown) or Ci protein detected by Ci-CT antibody (Fig. 2e) or Ci-NT antibody (not shown). PKA must therefore have a second target relevant to Hh signaling that does not influence Ci protein levels.

Although PKA hyperactivity did not discernibly affect Ci staining in cells in the center of each Ci stripe that expressed *wg* and *ptc* ectopically, it did suppress the elevation of Ci-CT antibody staining normally elicited by Hh at the borders of each Ci stripe (Fig. 2c). Thus, in the absence of Hh, Ci-CT staining responds dramatically to reductions in PKA activity (Figs. 2d and 3c) but is insensitive to increased PKA activity.

Responses to elevated PKA activity require *Smo* and *Ci* but not *Hh*

To explore the positive actions of PKA on Hh target gene expression further we examined the effects of various Hh pathway mutations. Loss of Hh activity did not impair the ectopic induction of *wg* and *ptc* by elevated PKA activity in alternating segments of *alt-mC** embryos (Fig. 4a–d). In contrast, Smo activity was required to observe any response of *wg* or *ptc* to elevated PKA even in embryos deficient only for zygotic *smo* (Fig. 4e,f). The essential activity of Smo, as opposed to Hh, cannot be explained by effects on Ci protein levels, as zygotic *smo* mutations and *hh* mutations have equivalent effects, reducing Ci-CT antibody staining only at the borders of each Ci stripe (Fig. 2b,g,h). The absolute requirement for Smo to observe transcriptional induction by PKA hyperactivity is consistent with two mechanisms. Either PKA acts on Smo, directly or indirectly, perhaps to uncouple it from the inhibitory influence of Ptc (Fig. 6, B and A, below) or, alternatively, Smo has Hh-independent activity that acts in parallel with PKA to stimulate *wg* and *ptc* expression (Fig. 6, C and A, below). Although the response to PKA hyperactivity did not involve changes in Ci staining it did absolutely require Ci activity (Fig. 4g,h), indicating a mechanism that either involves modification of Ci or requires Ci to act as a cofactor for *wg* and *ptc* expression.

Figure 4. Induction of *wg* and *ptc* by elevated PKA activity requires Smo and Ci but not Hh. (a,b) *alt-mC** (= *prd-GAL4* + *UAS-mC**) embryos show expanded *wg* RNA (a) and *ptc* RNA (b) expression in the even parasegments. (c,d) *alt-mC** embryos carrying the *hh^{ts2}* mutation lose wild-type stripes of *wg* RNA (c) and *ptc* RNA (d) in odd-numbered parasegments by stage 10, but expanded *wg* and *ptc* expression in cells with increased PKA activity is unaffected by the *hh* mutation. In *alt-mC** embryos that also lack maternal and zygotic *smo* (*smo^{11x43}*) (e,f) or zygotic *ci* activity [*Df(4)M62f* or *ci^{DR50}*] (g,h), *wg* RNA (e,g), and *ptc* RNA (f,h) expression are lost in all parasegments by stage 10, indicating that the responses to elevated PKA activity require *smo* and *ci* activity. In *alt-mC** embryos deficient only for zygotic *smo* (not shown), *wg* and *ptc* RNA expression decay at equal rates in adjacent segments and are lost by the end of stage 11.

Hh signaling does not require cAMP-mediated regulation of PKA activity

The structure of Smo has invited speculation that it couples to a G protein and might therefore regulate cAMP concentration (Alcedo et al. 1996; van den Heuvel and Ingham 1996). To examine this idea we tested whether low-level expression of mC* from an *actin-5C* promoter could substitute for *Drosophila* PKA catalytic subunit *DC0* activity in embryos. The mC* catalytic subunit binds poorly to regulatory subunit and is therefore active regardless of cAMP concentration (Orellana and McKnight 1992). Expression of mC* in the female germ line rescued the functions of *DC0* in oogenesis and allowed a small proportion of eggs to be fertilized and to develop (Y. Zhang, W. Li, and D. Kalderon, unpubl.). Many of these embryos hatched even if maternally and zygotically null for *DC0* and exhibited ventral cuticle patterns similar to wild type (Fig. 5a,b), implying normal Hh signaling. Hence, as observed previously in imaginal discs (Jiang and Struhl 1995; Li et al. 1995), neither transcriptional nor cAMP-dependent regulation of PKA activity appear to be essential for Hh signaling.

Discussion

Previous models of Hh signaling in *Drosophila* have emphasized changes in the concentration of Ci proteins, detected by Ci-CT antibody, as the goal of signal transduction. Also, the antagonistic effects of PKA on Hh target gene expression and involvement of the serpentine transmembrane protein Smo, predicted to couple to a G-protein, have sometimes been rationalized as indicative of cAMP acting as a key intermediate. We have shown that regulation of Ci protein levels is a common focus for the antagonistic actions of Hh and PKA in *Drosophila* embryos but have also obtained evidence for novel positive actions of both PKA and Hh in inducing Hh target genes without affecting Ci protein levels. In addition, we have shown that, as in imaginal discs, regulation of PKA activity by cAMP is not essential to Hh signal transduction in embryos.

Repression of Hh target genes by PKA; possible targets

PKA inhibition in *Drosophila* embryos induced ectopic expression of the Hh target genes *wg* and *ptc* (Fig. 1) and was accompanied by increased Ci-CT antibody staining, without affecting Ci-NT antibody staining or *ci* mRNA levels (Fig. 2). Because Hh has similar effects and has been shown to inhibit the cleavage of full-length Ci protein (Ci-155), it is very likely that PKA inhibition also reduces cleavage of Ci-155. This hypothesis must be tested directly in the future but is consistent with our observation that increased PKA activity suppresses the

Figure 5. Regulation of PKA by cAMP is not essential for Hh signaling. Ventral cuticles (fifth and sixth abdominal segments) of wild-type larvae (a) and larvae that expressed mC* from an *actin-5C* promoter but were maternally and zygotically null for *DC0* (b) showed the same trapezoidal pattern, number, and spacing of rows, indicating normal Hh signaling. In *ubi-R** larvae (PKA inhibition) (c) and *ubi-mC** larvae (PKA hyperactivity) (d) the wide posterior rows of each segment are replaced by narrower and often incomplete rows of different denticle morphology and polarity to give a rectangular denticle pattern characteristic of embryos expressing Hh ubiquitously at low levels. They do not show a duplication of the most anterior row in place of the posterior rows that is characteristic of *ptc* mutations.

duced only a slight induction of ectopic *ptc* expression and no ectopic induction of *wg* expression (Fig. 3a,b).

Despite the potent effects of PKA on Hh target gene expression, changes in cAMP concentration cannot account for Hh signaling. First, in embryos, as in imaginal discs, replacement of the major *Drosophila* PKA catalytic subunit DC0 with mC*, which is insensitive to cAMP, did not prevent Hh signaling (Jiang and Struhl 1995; Li et al. 1995). Second, neither inhibition nor activation of PKA in embryos stimulated Ci-CT antibody staining and *wg* RNA in the same proportion as normally induced by Hh. These observations do not exclude the possibility that modulation of PKA activity might make some contribution to Hh signaling, but they are not consistent with a central role for cAMP as a mediator of Hh signaling.

Dual actions of Hh signaling pathway

Prior to this study the only significant indicator that Hh signaling in *Drosophila* may involve more than simply regulating Ci protein levels were phenotypes attributable to *fu* mutations in which Hh target gene expression was attenuated or eliminated despite extremely high levels of Ci-CT antibody staining (Motzny and Holmgren 1995; Slusarski et al. 1995). Whether this activity of Fu reflected an activity of Hh was unclear, however, as Fu, although phosphorylated in response to Hh (Thérond et al. 1996b), is not required for Hh signaling in the absence of Su(fu) (Thérond et al. 1996a). In this study, we used PKA inhibition as a means of substituting for the effect of Hh on Ci protein processing. The increase in Ci-CT antibody staining because of PKA inhibition was at least as great as induced during normal Hh signaling and was not accompanied by any change in Ci-NT antibody staining, suggesting that PKA inhibition does phenocopy the effects of Hh on Ci cleavage. Under these circumstances, we found that both Hh and Smo were essential to maintain *wg* expression despite unchanged Ci staining. Thus, both Hh and Smo, an established mediator of Hh signals, provided a positive input to *wg* expression that was independent of detectable changes in Ci protein levels (Fig. 6A).

It was shown recently that in embryos with normal PKA activity *wg* can be induced by overexpression of a *ci* cDNA even in the absence of Hh activity (Alexandre et al. 1996). It is likely that we observed stricter requirements for Hh activity to induce *wg* expression because we simultaneously reduced the positive input from PKA toward Hh target gene expression (Fig. 6, B or C).

Functional implications of dual actions of PKA and Hh

We believe that the novel mechanisms depicted in Figure 6, namely a positive action of PKA and an action of Hh that is not related to alteration of Ci protein levels, are also operative in imaginal discs. In wing discs most patterning responses to Hh and the expression of *ptc* reveal only an antagonistic effect of PKA (Jiang and Struhl 1995; Lepage et al. 1995; Li et al. 1995; Pan and Rubin

1995). However, we have found that formation of non-neuronal bristles at the wing margin in response to either Hh or loss of *ptc* function requires PKA activity (J.T. Ohlmeyer, W. Li, and D. Kalderon, unpubl.), suggesting conservation of the positive function of PKA in Hh signaling. A similar defect in patterning of wing margin bristles is seen as a consequence of reduced Fu activity even though Ci-CT antibody staining is high in *fu* mutant cells responding to Hh (Slusarski et al. 1995; Sánchez-Herrero et al. 1996; J.T. Ohlmeyer, W. Li, and D. Kalderon, unpubl.). These observations suggest that the novel activities of Hh and PKA that we have described are general features of Hh signaling in *Drosophila* that are particularly obvious in embryos. It is possible that in vertebrates, as in *Drosophila* imaginal discs, a positive contribution of PKA to a subset of Hh responses is also present but not easily discerned in the background of the more prominent repressive effect of PKA on Hh target gene expression (Fan et al. 1995; Concordet et al. 1996; Epstein et al. 1996; Hammerschmidt et al. 1996; Ungar and Moon 1996).

The target specificity of the two arms of Hh signaling (Fig. 6) need not be identical even though the common requirement for Ci activity suggests that a Ci-binding site will always be present in target genes. In embryos we observed a differential dependence of *wg* and *ptc* expression on the novel arm of Hh signaling (Fig. 6A) by assaying the response to Hh at constant Ci protein levels (Fig. 3e,h). Similarly, in imaginal discs *fu* mutations allow Hh-dependent changes in Ci protein levels but have a greater inhibitory effect on the induction of anterior *en* expression than on the induction of *decapentaplegic* (*dpp*) expression by Hh (Slusarski et al. 1995; Sánchez-Herrero et al. 1996; J.T. Ohlmeyer, W. Li, and D. Kalderon, unpubl.). Also, productive signaling in each branch of the pathway might require a different threshold concentration of Hh. Hence, dual arms of Hh signaling provide a mechanism that may underlie the ability of Hh family molecules to produce different transcriptional responses in similar cells according to Hh concentration (Heemskerk and DiNardo 1994; Roelink et al. 1995; Ericson et al. 1996; Struhl et al. 1997) or the presence of other signaling molecules (Hooper 1994; Yoffe et al. 1995).

Materials and methods

Fly stocks and crosses

Flies homozygous for *UAS-R** and *UAS-mC** transgenes (Li et al. 1995) were crossed to flies homozygous for the "E22C" enhancer-trap line (A. Brand and N. Perrimon, unpubl.) or a *prd-GAL4* transgene, "RG1" (Yoffe et al. 1995) to generate embryos with reduced or excess PKA activity, respectively (confirmed by measurements in extracts in the presence of 1 μ M cAMP as described previously (Li et al. 1995). In all cases, embryos were collected for 3 hr at 25°C and incubated at 29°C for an additional 5 hr to obtain stage 10–13 embryos and to maximize expression of GAL4-responsive transgenes. *hh* mutations were introduced by crossing *UAS-R** or *UAS-mC**; *hh^{ts2}*/TM6B flies to *hh^{ts2}* RG1/TM6B flies. Homozygous *hh^{ts2}* progeny were identified by loss of *wg*, *ptc*, or En (in double stains with Ci) expres-

sion in alternate segments and were found in the expected proportions. *hh^{ts2}* is a hypomorph reported to have no discernible activity at 29°C (Ma et al. 1993). Embryos zygotically mutant for *smo^{11x43}* were similarly identified among progeny of the crosses *smo^{11x43} UAS-mC*/CyO* or *smo^{11x43}/CyO; UAS-R*/UAS-R* × smo^{11x43}/CyO; RG1/RG1* (or *E22C smo^{11x43}/CyO*). Embryos also maternally deficient for *smo* were generated by the FLP-dominant female sterile (DFS) technique (Chou et al. 1993), inducing germ-line clones in females that were *smo^{11x43} FRT40A/P[ovo^{D1}] FRT40A; UAS-R*/+* or *smo^{11x43} FRT40A UAS-mC*/P[ovo^{D1}] FRT40A* and crossing to *smo^{11x43}/CyO; RG1/RG1* males. *smo^{11x43}* is amorphic (van den Heuvel and Ingham 1996). The effects of *ci* mutations were tested by crosses between *UAS-R*/UAS-R** or *UAS-mC*/UAS-mC**; *Df(4)M62f/ey^D* or *ci^{DR50}/M63a* females and *E22C/E22C* or *RG1/RG1; Df(4)M62f/ey^D* or *ci^{DR50}/M63a* males. *M62f* is a deficiency that is null for *ci* and *ci^{DR50}* is a loss-of-function mutation (Slusarski et al. 1995); identical results were found with each allele. *DC0* null germ-line clones (Lane and Kalderon 1994) were generated at the same time as activating *mC** expression from an *actin-5C* promoter by inducing FLP recombination in females that were *DC0^{H2} FRT40A actin <y⁺ <mC*/P[ovo^{D1}] FRT40A* (where < denotes an FRT) to produce the germ-line genotype *DC0^{H2} FRT40A actin <mC*/DC0^{H2} FRT40A* and crossing to *Df(2L)γ15/P[y⁺] CyO* males heterozygous for a *DC0* deficiency. Cuticles of hatching *y⁺* larvae were examined.

Examination of embryos

In situ hybridizations on whole-mount embryos used digoxigenin-labeled RNA probes synthesized by T7 RNA polymerase for *wg*, plasmid *wgPx4* (J. Mohler, Barnard College, New York, NY) cut by *Pst*I; *ptc*, plasmid *Dra2Nptc* (S. DiNardo, Rockefeller University, New York, NY) cut by *Hinc*II; *hh*, plasmid *4.1/8B#6* (J. Mohler) cut by *Nde*I; and *ci*, plasmid *pGEM7Zci* (R. Holmgren, Northwestern University, Evanston, IL) cut by *Bam*HI and *Xba*I. Antibody stainings were performed using monoclonal En/Inv antibody [4D9; (Patel et al. 1989)], monoclonal Ci-CT antibody [2A1; (Motzny and Holmgren 1995)], polyclonal Ci-NT antibody (Aza-Blanc et al. 1997), or polyclonal anti-β-galactosidase (Cappel). Larval cuticles were prepared by a modification (S. DiNardo) of a published procedure (Heemskerk and DiNardo 1994).

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