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Drosophila *Costal1* Mutations Are Alleles of Protein Kinase A That Modulate Hedgehog Signaling

Lara S. Collier,[1] Kaye Suyama, Joseph H. Anderson[2] and Matthew P. Scott[3]

Program in Cancer Biology, Departments of Developmental Biology and Genetics, Howard Hughes Medical Institute, Stanford University School of Medicine, Stanford, California 94305-5439

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ABSTRACT

Hedgehog (Hh) signaling is crucial for the development of many tissues, and altered Hh signal transduction can result in cancer. The Drosophila *Costal1* (*Cos1*) and *costal2* (*cos2*) genes have been implicated in Hh signaling. *cos2* encodes a kinesin-related molecule, one component of a cytoplasmic complex of Hh signal transducers. Mutations in *Cos1* enhance loss-of-function *cos2* mutations, but the molecular nature of *Cos1* has been unknown. We found that previously identified alleles of *Cos1* actually map to two separate loci. Four alleles of *Cos1* appear to be dominant-negative mutations of a catalytic subunit of protein kinase A (*pka-C1*) and the fifth allele, $Cos1^{A1}$, is a gain-of-function allele of the PKA regulatory subunit *pka-RII*. PKA-RII protein levels are higher in $Cos1^{A1}$ mutants than in wild type. Overexpression of wild-type *pka-RII* phenocopies *Cos1* mutants. PKA activity is aberrant in $Cos1^{A1}$ mutants. PKA-RII is uniformly overproduced in the wing imaginal disc in $Cos1^{A1}$ mutants, but only certain cells respond by activating the transcription factor Ci and Hh target gene transcription. This work shows that overexpression of a wild-type regulatory subunit of PKA is sufficient to activate Hh target gene transcription.

THE secreted signaling molecule Hedgehog (Hh) was originally discovered as a gene required for proper patterning of the Drosophila larval cuticle and is now known to function in many different tissues at many different stages of Drosophila development. Hh signaling has been highly conserved during evolution and is employed in the development of many mammalian tissues and organs as well. Human cancers, including basal cell carcinoma, medulloblastoma, and digestive tract tumors, occur when the Hh signal is overproduced or restraining influences on Hh signal transduction are damaged (Hahn *et al.* 1996; Johnson *et al.* 1996; Goodrich *et al.* 1997; Oro *et al.* 1997; Raffel *et al.* 1997; Taipale and Beachy 2001; Berman *et al.* 2003; Thayer *et al.* 2003). During Drosophila larval development, Hh is expressed and secreted by cells in the posterior (P) of developing imaginal discs. Anterior (A) cells respond to Hh by activating transcription of target genes in cells just anterior to the A/P compartment boundary. In the wing imaginal disc these transcriptional targets include *patched* (*ptc*), *engrailed* (*en*), and the transforming growth factor β (TGFβ) family member *decapentaplegic* (*dpp*; Basler and Struhl 1994; Capdevila and Guerrero 1994). Dpp itself is a secreted signaling molecule that functions as a morphogen to pattern the wing (Zecca *et al.* 1995; Nellen *et al.* 1996). When too little Hh signaling occurs in the wing and *dpp* expression is attenuated, structures at the A/P boundary are lost (Johnson *et al.* 1995). Conversely, if Hh signaling is activated inappropriately and ectopic *dpp* is produced, overgrowth and duplications of the anterior compartment occur (Capdevila *et al.* 1994).

Anterior cells must efficiently activate a transcriptional program in response to the Hh signal and must also ensure that Hh target genes are not activated inappropriately in the absence of ligand. Loss of any of several negative regulators in the Hh signal transduction pathway gives rise to Hh target gene activation in the absence of Hh. These include the 12-pass transmembrane protein Patched (Ptc; Hooper and Scott 1989; Nakano *et al.* 1989), the kinesin-related protein Costal2 (Cos2; Robbins *et al.* 1997; Sisson *et al.* 1997), the C1 catalytic subunit of the serine-threonine kinase protein kinase A (PKA-C1; Jiang and Struhl 1995; Johnson *et al.* 1995; Lepage *et al.* 1995; Li *et al.* 1995; Pan and Rubin 1995), and the Cdc4-like protein Supernumerary limbs (Slimb; Jiang and Struhl 1998; Theodosiou *et al.* 1998). Recent experiments have uncovered additional potential *positive* roles for *pka-C1* and *cos2* in regulating Hh target gene transcription (Wang and Holmgren 2000).

Like *cos2*, *Costal1* (*Cos1*) mutants result in phenotypes similar to *hh* gain of function (Figure 1; Whittle 1973, 1974; Grau and Simpson 1987), so mutant alleles of

[1]*Present address:* Department of Genetics, Cell Biology, and Development, University of Minnesota, Minneapolis, MN 55455.

[2]*Present address:* School of Aquatic and Fishery Sciences, University of Washington, Seattle, WA 98195.

[3]*Corresponding author:* Clark Center W252, 318 Campus Dr., Stanford University School of Medicine, Stanford, CA 94305-5439. E-mail: scott@cmgm.stanford.edu

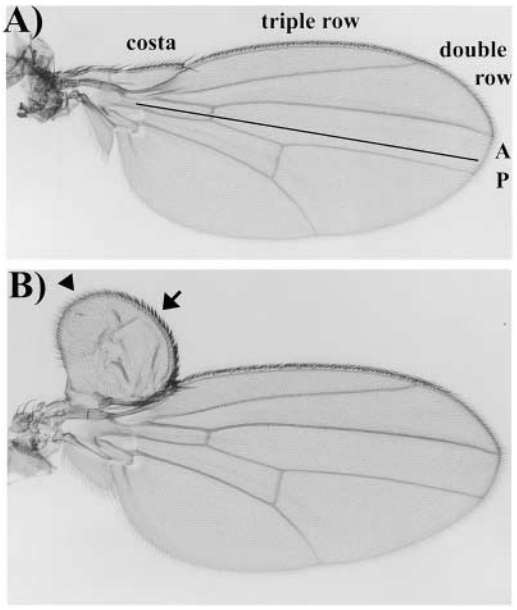


Figure 1.—The *Cos1* phenotype. (A) A wild-type (Canton-S) male wing. The A/P boundary runs just anterior to vein IV and is indicated by a diagonal line. Anterior structures include the costa, the triple-row bristles, and the double-row bristles. (B) A *b pr cos2^{V1}cn Cos1^{A1}*/+ mutant male wing displaying a large duplication of the costa region. The duplication consists of anterior structures as it contains triple-row (arrow) and double-row (arrowhead) bristles.

Cos1 are likely to allow inappropriate transcription of Hh target genes (Capdevila *et al.* 1994). The role of the zinc-finger transcription factor Cubitus interruptus (Ci) in Hh signal transduction is complex as it functions as both a negative and a positive regulator of transcription (Aza-Blanc *et al.* 1997).

The genetic data show that *ptc*, *cos2*, and *pka-C1* are negative regulators of Hh target gene transcription. Unlike *ptc* and *cos2*, which appear to function exclusively in Hh signaling, *pka-C1* plays many roles in addition to controlling transduction of the Hh signal. For example, *pka-C1* is necessary for Drosophila oogenesis and neural function, as indicated by studies of *pka-C1* hypomorphic alleles (Lane and Kalderon 1993; Skoulakis *et al.* 1993). Later analysis of *pka-C1* null alleles in clones of cells revealed wing duplications and other patterning defects that linked *pka-C1* to Hh signal transduction (Jiang and Struhl 1995; Johnson *et al.* 1995; Lepage *et al.* 1995; Li *et al.* 1995; Pan and Rubin 1995).

Pioneering studies of PKA showed how it is regulated by cAMP. In the absence of cAMP, PKA exists as a heterotetramer consisting of two catalytic (C) subunits and a homodimer of two regulatory (R) subunits, and the enzyme is inactive. Upon binding of two cAMP molecules to each R subunit, the C subunits are released as catalytically active monomers. Drosophila has two R subunits, RI and RII (Kalderon and Rubin 1988; Park *et al.* 2000). The ability of R subunits to influence PKA-C1 activity in the context of Hh signaling has been explored only by overexpression of a mutant RI subunit that cannot bind cAMP, which mimics *pka-C1* loss-of-function phenotypes (Li *et al.* 1995; Kiger *et al.* 1999). The role of wild-type R subunits in transducing the Hh signal is unknown.

Hh signal transduction culminates in the control of the activity of the transcription factor Ci, which can act as either a repressor or an activator of Hh target genes. In the wing imaginal disc, Hh signaling acts through Ci to activate the transcription of *dpp*, *ptc*, and *en*. In the absence of Hh, Ci is proteolytically processed into a 75-kD repressor form that represses *dpp*, but not *ptc* or *en* (Methot and Basler 1999). PKA affects Hh signal transduction by phosphorylating Ci and thus promoting processing of Ci into the repressor form (Chen *et al.* 1998, 1999; Price and Kalderon 1999; Wang *et al.* 1999). It is unknown how the Hh signal either prevents PKA from phosphorylating Ci or activates an opposing phosphatase. Whether PKA also affects Hh signaling in other ways is unknown.

In previous work the *Cos1* gene has been mapped to at least two locations, suggesting that similar genetic effects derive from more than one locus (Grau and Simpson 1987; Lasko and Pardue 1988). Here we have found that certain *Cos1* alleles map to two loci, both of them encoding subunits of PKA. Our genetic studies show that different *Cos1* alleles affect Hh signal transduction in distinct ways.

MATERIALS AND METHODS

Fly stocks: *P*-element lines were obtained from Bloomington Stock Center, Zieged Stock Center, and Exelixis. *JW1 Gal4* was a gift of J. Kiger. For *Cos1^{A1}* heterozygous discs, *BS3.0 dpp LacZ* (Blackman *et al.* 1991) was used. For *Cos1^{A1}* clonal analysis, a line carrying *dpp LacZ* on the third chromosome was used (Glise *et al.* 2002).

Mounting wings: Wings were dissected in ethanol and mounted in a 1:1 solution of Permount and Xylene.

Recombination mapping: *w; b pr cos2^{V1} cn Cos1^{A1}/P, w; b pr cos2^{V1} cn Cos1^{2} bw/P, w; b pr cos2^{V1} cn Cos1^{3} bw/P, w; b pr cos2^{V1} cn Cos1^{8} bw/P*, and *w; b pr cos2^{V1} cn Cos1^{9} bw/P* virgin females were mated to *w; Sco/CyORoi* males. Eye-color and wing phenotypes of progeny were scored. Due to variable penetrance of the wing duplication for *cos2^{V1} Cos1* chromosomes, only red-eyed progeny were counted to determine map distance. All *P* elements used for mapping were marked with *miniwhite*+.

For *Cos1^{A1}* mapping experiments, stocks were established from recombinant progeny by crossing to *w, Sco/CyORoi*. DNA was made from the recombinant stocks using a Berkeley *Drosophila* Genome Project (BDGP) protocol (http://www.fruitfly.org/about/methods/inverse.pcr.html) and the presence or absence of *cos2^{V1}* on the recombinant chromosomes was determined by PCR.

***cos2^{V1}* genotyping PCR:** PCR was performed using HFtaq (BD Biosciences) with an annealing/extension temperature of 62°. A forward primer that recognizes both *cos2*+ and *cos2^{V1}* genomic sequences (complete sequence 5′-TGTGAAGCAA TAGCTCAGATCCTG-3′) along with a *cos2*+-specific reverse primer (5′-TCACACGCTGATATTGAGGGAAC-3′) and a *cos2^{V1}*-specific reverse primer (5′-TGACTCCGCTCATAATCC GTACAC-3′) were used. *cos2*+ and *cos2^{V1}* products could be

recognized due to a size difference (~650 bp for *cos2*+ *vs.* 400 bp for $cos2^{V1}$).

5′-rapid amplification of cDNA ends for $Cos1^{A1}$ sequences: RNA was isolated from dechorionated 0- to 7-hr collections of *b pr* $cos2^{V1}$ *cn* $Cos1^{A1}$*/CyO* or *b pr cn* $cos2^{V1}$ *bw sp* embryos using RNAZOL (Teltest). 5′-rapid amplification of cDNA ends (RACE) was performed according to manufacturer's protocol (Roche, Indianapolis). A primer directed at base pairs 628–607 of the RII mRNA (5′-TTGGTTCATCTGCTCCTTCTCG-3′) was used for 5′-tagged first-strand synthesis. One round of HFtaq PCR (BD Biosciences) was performed using a primer directed at base pairs 555–533 (5′-TGCTCATCTGTCTTGGG GAACAC-3′) of the RII mRNA and a 5′-tag-specific primer. No detectable product was obtained, so a 1:10 dilution of the reaction was made and used in a second round of PCR using a primer at base pairs 482–464 (5′-CGAAAACTGATTTGC GACG-3′) of the RII mRNA and a 5′-tag-specific primer. The resulting products were TA cloned (Invitrogen, San Diego) and sequenced.

Allele sequencing: Genomic DNA was isolated using a BDGP protocol (http://www.fruitfly.org/about/methods/inverse.pcr.html) or DNEasy kit (QIAGEN, Chatsworth, CA). PCR was performed using the HF Taq kit (BD Biosciences). PCR products were purified from agarose gels (QIAGEN) and subjected to automated sequencing.

To generate homozygous mutant DNA for sequencing, a *b pr* $cos2^{V1}$ *cn* $Cos1^{A1}$*/CKG19* (*CyO Kr-Gal4, UAS-GFP*) stock was used. Adults 0–2 days old were placed in collection bottles in the dark and provided with apple/agarose collection caps with yeast paste. Caps were changed twice a day for 2 days. On the third and subsequent days, two 1-hr prelays were discarded. A 2-hr collection was then aged an additional 4.5 hr at room temperature. The resulting 4.5- to 6.5-hr embryos were dechorionated and hand sorted. Non-green fluorescent protein (GFP)-containing embryos were used to make genomic DNA. Purity of the sort was determined by PCR for *cos2*+ and $cos2^{V1}$. Only sorted pools that had no detectable PCR product for *cos2*+ were used for further analysis.

Preparation of protein extract from homozygous $Cos1^{A1}$ embryos for Western analysis and kinase assays: Homozygous *b pr* $cos2^{V1}$ *cn* $Cos1^{A1}$ embryos were obtained as previously described in *Allele sequencing* except that collections were sorted using automated methods (Furlong *et al.* 2001). Purity of the sort was determined by examining sorted embryos for GFP. Sorted embryos were incubated for 10 min on ice in hypotonic lysis buffer [10 mm Tris, pH 7.4, 0.2 mm MgCl2, and complete mini protease inhibitor (Roche)] and then dounce homogenized. Sucrose and EDTA were added to final concentrations of 0.25 m and 1 mm, respectively. Extracts were spun at 100,000 × *g* for 1 hr at 4°. The protein concentration of the resulting S100 was determined in triplicate using a Bradford Assay (Bio-Rad, Richmond, CA). For Western analysis, rabbit anti-RII sera were used at 1:2000. Kinase assays were performed using the SignaTECT PKA assay system (Promega, Madison, WI). cAMP concentrations were varied. Reactions were performed for 10 min at 30°. Specific activity was determined by subtracting from each experimental condition the counts incorporated in the presence of 1 μm of the PKA inhibitor-(6-22)-amide (see supplementary Figure 3 at http://www.genetics.org/supplemental/). Each assay was performed in triplicate. Wild type *vs.* mutant was compared at each concentration of cAMP using Student's *t*-test.

Clonal analysis: A *G13 FRT 42B* $Cos1^{A1}$ chromosome was generated, recombined with wild-type chromosomes for seven generations, and then balanced over *CyO*. A cross of *y,w, hsFLP; G13 FRT 42B* $Cos1^{A1}$*/CyO* virgins to *y,w, hsFLP; G13 FRT Ubi-GFPnls* males was allowed to lay for 2 days. Larvae were heat-shocked at 37° for 1 hr on the third and fourth days or on the fourth and fifth days.

Antibody staining of imaginal discs: Third instar larvae were dissected in PBS and fixed in Brower's fix for 1 hr on ice or in 4% paraformaldehyde in PBS for 13 min at room temperature. Larvae were washed in PBS, 0.1% Tween, and 0.2% BSA. Primary antibodies were hybridized in wash buffer overnight at 4°. Larvae were washed and secondary antibodies were hybridized at room temperature for 1 hr. After additional washes, discs were mounted in Vectashield and imaged using confocal microscopy. Primary antibody concentrations were as follows: rabbit polyclonal anti-RII, 1:2000; rat monoclonal anti-Ci Cterm (2A1), 1:10; rabbit polyclonal and mouse anti-β-gal (Promega), 1:5000.

Overexpression studies: *JW1 Gal4* was crossed to *EP(2)2162* or *EP(2)2277* as a control. All crosses were carried out at 29°.

RESULTS

***$Cos1^2$*, *$Cos1^3$*, *$Cos1^8$*, and *$Cos1^9$* encode dominant-negative alleles of *pka-C1*:** Two conflicting map locations have been published for *Cos1*. $Cos1^{A1}$, $Cos1^2$, and $Cos1^3$ were mapped by meiotic recombination to a position distal to *cn* and proximal to *vg* on the right arm of the second chromosome (2R). Two other alleles, $Cos1^8$ and $Cos1^9$, were not mapped but were considered to be allelic to *Cos1* because of their dominant wing phenotype and lethality when tested *in trans* to other *Cos1* alleles (Grau and Simpson 1987). *Cos1* was later placed at a different map location on 2R distal to *vg* at 50A1–50A2. This map location was based on the discovery of a deficiency in the region that produced duplications when placed *in trans* to a recessive allele of *cos1* (Lasko and Pardue 1988).

To resolve this discrepancy, meiotic map locations were determined for all dominant alleles of *Cos1*. This was accomplished by measuring map distances between *Cos1* alleles and individual *white*$^+$ (w^+) *P*-element insertions. *Cos1* results in dominant wing duplications and the *P* element results in dominant eye pigmentation in a *w* background, so recombinant progeny can be scored in the first generation by scoring wing and eye phenotypes. Because of variable penetrance of *Cos1* (which can range from 3 to 95%, depending on the allele and positive or negative selection for the phenotype; Grau and Simpson 1987), some recombinant progeny will not be scored as such and thus recombination percentages are underestimations of map distance. In addition, only w^+ progeny were scored for calculating map distances.

The previously unmapped allele $Cos1^9$ was mapped using a panel of *P* elements on the second chromosome. Surprisingly, $Cos1^9$ recombined readily with all *P*-transposable elements that were located on 2R, indicating that $Cos1^9$ does not map to either previously published map location for *Cos1* (supplementary Figure 1 at http://www.genetics.org/supplemental/). For *P* elements on chromosome arm 2L, recombination percentages with $Cos1^9$ decreased as *P* elements farther and farther from the centromere were used, until polytene band 30C, indicating that $Cos1^9$ maps close to polytene band 30C. This was confirmed by the lack of recombination be-

tween *Cos1*^{9} and the *k07104 P*-element insertion at 30C. With *P* elements distal to 30C, recombination percentages increased with *P* elements closer and closer to the telomere of chromosome arm 2L (supplementary Figure 1 at http://www.genetics.org/supplemental/). *Cos1*^{2} also mapped to this location (data not shown). *Cos1*^{3} and *Cos1*^{8} were difficult to map due to low penetrance, yet both failed to recombine with the *k07104 P* element, indicating a map location near 30C (data not shown).

Inspection of the 30C region to which *Cos1*^{2}, *Cos1*^{3}, *Cos1*^{8}, and *Cos1*^{9} mapped revealed the presence of the gene encoding the protein kinase A C1 subunit (*pka-C1*). Because loss of *pka-C1* function activates Hh target gene transcription (Jiang and Struhl 1995; Li *et al.* 1995; Strutt *et al.* 1995), there was reason to suspect that *pka-C1* could be the *Cos1* gene located near 30C. The *pka-C1* protein-coding sequence was sequenced using DNA obtained from *Cos1*^{2}, *Cos1*^{3}, *Cos1*^{8}, and *Cos1*^{9} heterozygous flies and point mutations were found in the gene in each of the four stocks. *Cos1*^{2} and *Cos1*^{9} each contained the same base-pair substitution that translated into a change in protein sequence of E to K at amino acid (aa) 130. *Cos1*^{3} contained a base-pair substitution that translates to an E-to-K change at aa 173 and *Cos1*^{8} contained a base-pair change that translates to a G-to-D substitution at aa 189 (Figure 2). These changes are not present in wild-type Canton-S flies or on the CyO balancer chromosome. All three residues are conserved from yeast to human.

On the basis of these data, *Cos1*^{2}, *Cos1*^{3}, *Cos1*^{8}, and *Cos1*^{9} should be renamed *pka-C1*$^{Cos1–2}$, *pka-C1*$^{Cos1–3}$, *pka-C1*$^{Cos1–8}$, and *pka-C1*$^{Cos1–9}$.

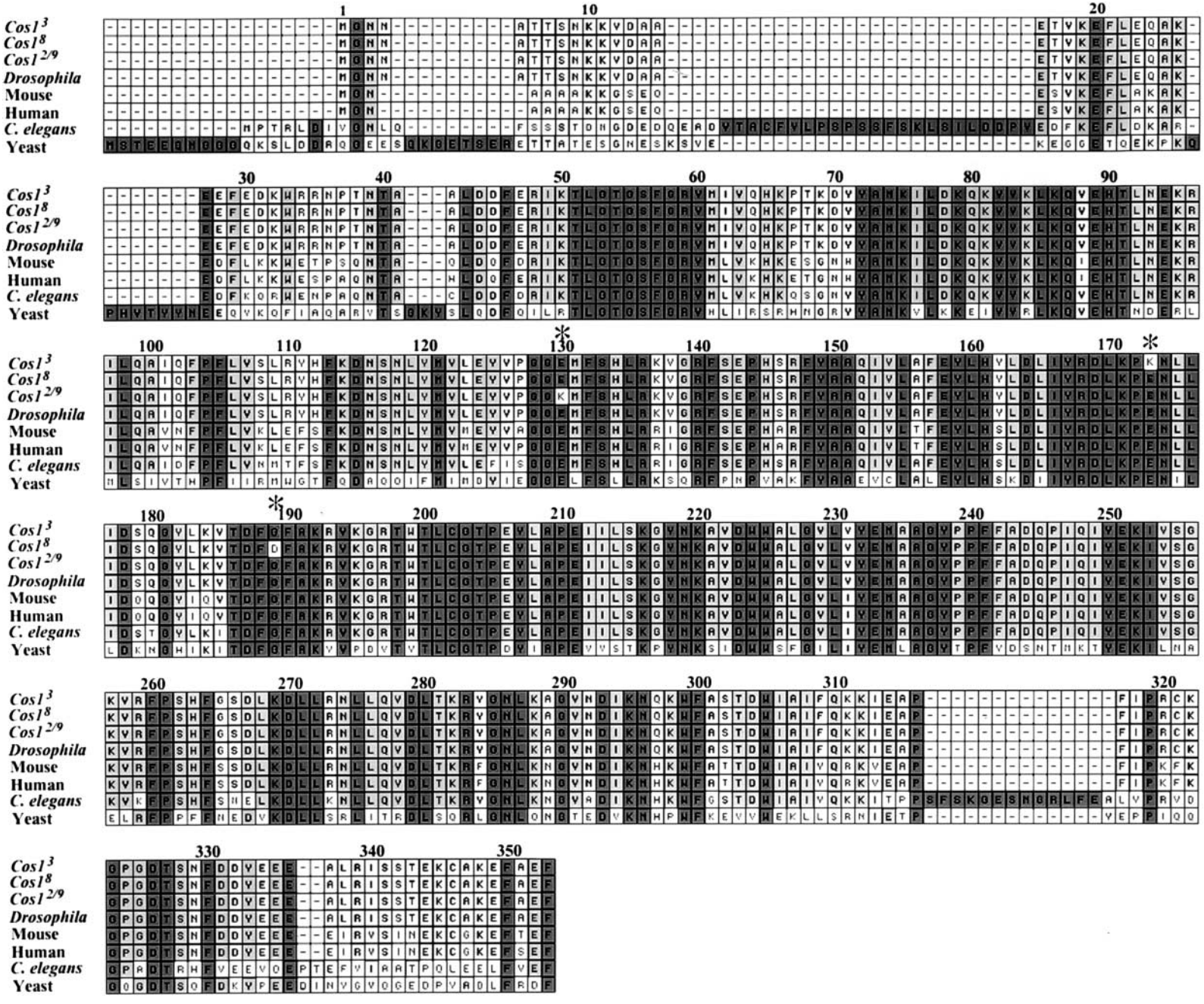
***Cos1*^{A1} encodes a gain-of-function allele of *pka-RII*:** Unlike the other *Cos1* alleles, *Cos1*^{A1} did not recombine readily with *P* elements in the *cn* to *vg* region of chromosome 2R (supplementary Figure 2 at http://www.genetics.org/supplemental/). Specifically, *Cos1*^{A1} recombination percentages with *P*-element insertion *k13906* at 46D1 and with *P*-element insertion *EP(2)2170* at 46E1 were 0.05%, indicating that *Cos1*^{A1} maps near these two *P* elements. Because the *Cos1*^{A1} chromosome used for mapping is also marked with an allele of *cos2*, *cos2*^{V1}, the linear order of the centromere, *Cos1*^{A1}, and the *P* element could be determined for each recombinant chromosome. *cos2*^{V1} and *Cos1*^{A1} are linked (they are separated by ~5 MU), and the gene order is centromere, *cos2*^{V1}, *Cos1*^{A1}, telomere (Grau and Simpson 1987). Recombination events between *Cos1*^{A1} and the *P* element would result in recombinant chromosomes that either include or exclude *cos2*^{V1}, depending on the proximal/distal relationship of *Cos1*^{A1} and the *P* element. The presence of *cos2*^{V1} on a *Cos1*^{A1} *P*-element recombinant chromosome would indicate that *Cos1*^{A1} is proximal to the *P* insertion. Conversely, the absence of *cos2*^{V1} would indicate that *Cos1*^{A1} is distal to the *P* insertion.

cos2^{V1} contains a transposon insertion in the 3′-untranslated region (UTR) of *cos2* (Sisson *et al.* 1997; data not shown) so the presence of *cos2*^{V1} can be easily elucidated by PCR using a primer in *cos2* and a primer specific to the insert. Using this method, *Cos1*^{A1} was found to be distal to *P*-element insertion *k13906* at polytene band 46D1 and proximal to *P*-element insertion *EP(2)2170* at polytene 46E4 (data not shown). No recombinants were detected between *Cos1*^{A1} and *P*-element insertion *EP(2)2162* at polytene band 46D1 (n = 6640), *P*-element insertion *EP(2)2324* at polytene band 46D7 (n = 5935), and *P*-element insertion *EP(2)2277* at polytene band 46D7 (n = 9776; supplementary Figure 2 at http://www.genetics.org/supplemental/). The mapping of *Cos1*^{A1} to polytene chromosome location 46D–E is in agreement with the previously identified meiotic map location (61) published by Grau and Simpson (1987).

Examination of the sequence within the maximal chromosome region that must contain at least part of *Cos1*^{A1} (between *P* element *k13906* and *P* element *EP(2)2170*, an interval of 156 kb) revealed 26 genes predicted by the Drosophila genome project. One of these 26 genes encodes a regulatory subunit of protein kinase A, *pka-RII*. *pka-RII* was already known to influence PKA catalytic activity in adult Drosophila (Park *et al.* 2000), but the role of *pka-RII* in regulating PKA-C1 activity in Hh signaling has not been elucidated. Because *pka-RII* can regulate PKA catalytic activity in certain circumstances, it was a candidate for the *Cos1*^{A1} mutation.

Genomic DNA from homozygous mutant *Cos1*^{A1} embryos was isolated and used as a template for PCR reactions to amplify the *pka-RII* genomic region. When primers directed against a portion of the second intron of *pka-RII* were used, no product was obtained for *Cos1*^{A1} mutants, but the expected product was obtained from wild-type Drosophila DNA (data not shown). 5′ RACE was employed to determine the sequence of the *pka-RII* mRNA in *Cos1*^{A1} mutants. The mutant transcript contained the third exon of *pka-RII* (which contains the ATG) but the first and second exons were missing. In their place was sequence from the *14-3-3* and *JRA* genes, which are located ~100 kb distal to *pka-RII*. The se-

Figure 2.—*Cos1*^{2}, *Cos1*^{3}, *Cos1*^{8}, and *Cos1*^{9} contain amino acid substitutions in PKA-C1. A CLUSTALW alignment of *Cos1*^{2}/*Cos1*^{9}, *Cos1*^{3}, *Cos1*^{8}, and wild-type Drosophila PKA-C1 protein sequences is shown (Thompson *et al.* 1994). In addition to Drosophila PKA-C1, *Caenorhabditis elegans*, yeast, mouse, and human proteins are shown to indicate regions of conservation. Asterisks indicate amino acid substitutions in *Cos1* alleles. Darker background shading indicates a higher degree of conservation. *Cos1*^{2} and *Cos1*^{9} have an E-to-K substitution at amino acid 130. *Cos1*^{3} contains an E-to-K substitution at amino acid 173. *Cos1*^{8} possesses a G-to-D substitution at amino acid 189. All three residues are conserved from yeast to human.


Cos1[3]
Cos1[8]
Cos1[2/9]
Drosophila
Mouse
Human
C. elegans
Yeast
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10
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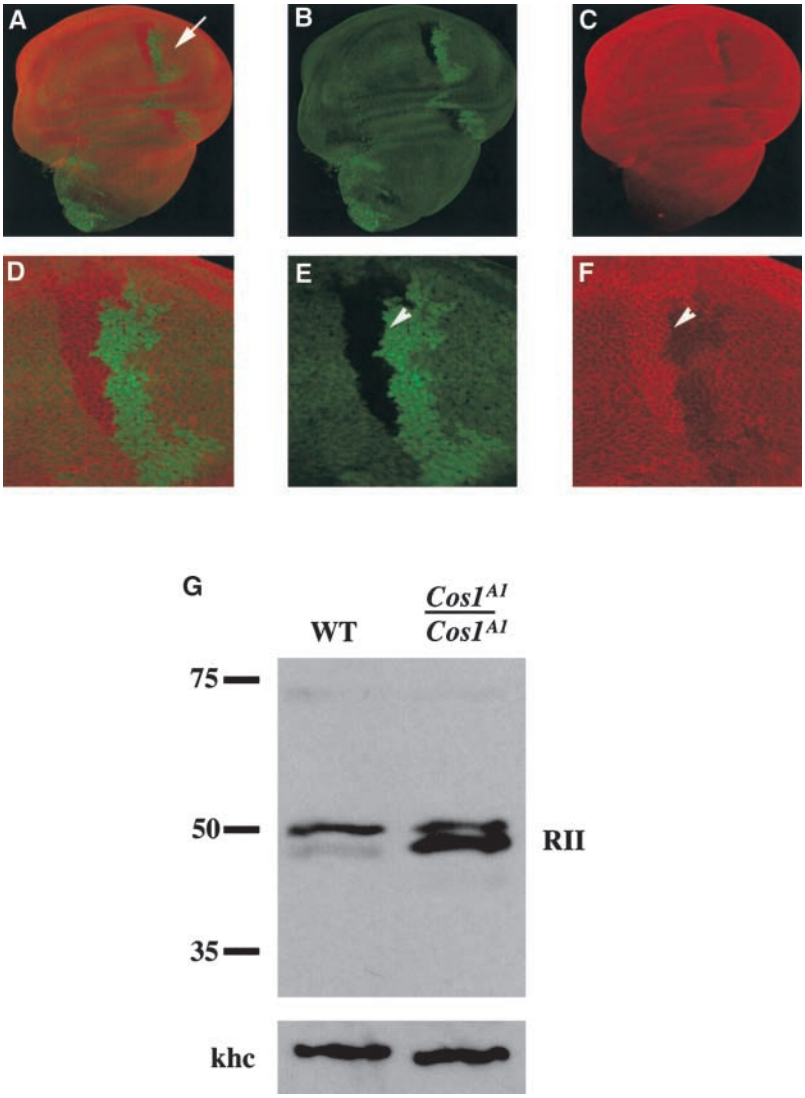


FIGURE 3.—RII protein levels are elevated in $Cos1^{A1}$ mutants. (A–F) $Cos1^{A1}$ clones were induced in wing imaginal discs. (A) A merged image of B and C. (B) Two copies of GFP mark homozygous wild-type cells (bright green) and lack of GFP marks $Cos1^{A1}$ homozygous mutant cells. (C) RII protein stain. (D–F) Detail of clone marked by arrow in A; arrowheads in E and F mark edges of $Cos1^{A1}$ homozygous mutant clones. RII levels are highest in $Cos1^{A1}$ homozygous mutant cells and intermediate levels of RII are detected in cells heterozygous for $Cos1^{A1}$. (G) Western blot for RII. Protein extract was made from wild-type (Canton-S) embryos or homozygous *b pr* $cos2^{V1}$ *cn* $Cos1^{A1}$ mutant embryos. RII antibody recognizes a doublet at ~50 kD. RII levels are elevated in mutant compared to wild type. An antibody against kinesin heavy chain was used to demonstrate that equal amounts of protein were loaded in each lane.

quence of the mutant mRNA is consistent with the underlying aberration being an inversion. No other changes were detected in the coding sequence for *pka-RII* in $Cos1^{A1}$ mutants.

To investigate the effect of the inversion on *pka-RII* expression, marked homozygous mutant clones of $Cos1^{A1}$ were generated in the wing imaginal disc using the flipase-flipase recombinase target system (HARRISON and PERRIMON 1993) and were stained with an antibody directed against PKA-RII (RII). In cells containing one mutant copy of $Cos1^{A1}$, a moderate level of staining for RII was observed. In cells wild-type for $Cos1^{A1}$, a low or background level of staining was observed. In homozygous $Cos1^{A1}$ cells, a high level of RII protein was detected (Figure 3). This result suggests that the *pka-RII* gene is the relevant gene for this *Cos1* allele and that the underlying mutation causes overproduction of the regulatory subunit of the PKA enzyme.

Cos1 mutant embryos have mutant phenotypes indicative of misregulation of Hh signaling during embryogenesis (GRAU and SIMPSON 1987). RII protein levels were assayed in extracts prepared from $cos2^{V1}$ $Cos1^{A1}$ homozygous mutant embryos to determine whether RII is also overproduced in $Cos1^{A1}$ mutants at early stages of development. For this purpose, homozygous $cos2^{V1}$ $Cos1^{A1}$ mutant embryos were obtained by automated sorting from a GFP-balanced stock (FURLONG *et al.* 2001). Compared to wild type, $Cos1^{A1}$ mutants contain very high levels of RII protein (Figure 3). Kinesin heavy chain, detected with a specific antibody, was used to demonstrate that equal amounts of total protein were present for each genotype. As previously reported, Drosophila RII migrates as a doublet with an apparent molecular weight of ~50 kD (PARK *et al.* 2000; Figure 3).

One explanation for the high levels of RII protein found in $Cos1^{A1}$ mutants is that *pka-RII* is a transcriptional target of Hh signaling. If this were the case, high levels of RII should be found in cells mutant for other negative regulators of Hh target gene transcription. To investigate this possibility, clones of homozygous *cos2* mutant

cells were generated and stained for RII. No increased staining for RII was detected in *cos2* clones (data not shown), indicating that *pka-RII* is not a transcriptional target of Hh.

To determine whether *pka-RII* overexpression is sufficient to phenocopy *Cos1* mutants, the GAL4-*u*pstream *a*ctivating *s*equence (UAS) system was used to overproduce RII in the developing wing. The transposable element *EP(2)2162* is inserted 5′ to the transcriptional start of *pka-RII* (Park *et al.* 2000). The enhanced promoter (EP) type of *P* elements contains UAS sites that allow GAL4-mediated overexpression of downstream genes (Rorth *et al.* 1998). *EP(2)2162* is in the correct orientation to drive overexpression of RII (Park *et al.* 2000). *JW1 Gal4* expresses Gal4 in the wing imaginal disc (Kiger *et al.* 1999; Kiger and O'Shea 2001) and was used to activate *pka-RII* transcription by taking advantage of the UAS element in *EP(2)2162*. Activation of *pka-RII* transcription resulted in outgrowths and duplications of the costa region of the wing, a *Cos1* phenotype. When *JW1 Gal4* was used to express *EP(2)2277*, a control EP element, no duplications were observed, indicating that the phenotype is not a result of GAL4 expression alone (Figure 4). Duplications also occurred when RII was overproduced with the *E132 Gal4* driver (data not shown).

In summary, a small interval that contains *pka-RII* and must also contain part of $Cos1^{A1}$ was identified by recombination mapping. In total, three lines of evidence support that *pka-RII* is the gene affected in $Cos1^{A1}$ mutants: (1) an inversion with a breakpoint in *pka-RII* was found in $Cos1^{A1}$ mutants, (2) RII is overproduced in $Cos1^{A1}$ mutants, and (3) overexpression of wild-type *pka-RII* is sufficient to mimic a $Cos1^{A1}$ phenotype. On the basis of these data, $Cos1^{A1}$ should be renamed $pka\text{-}RII^{Cos1A1}$.

PKA activity is affected in $pka\text{-}RII^{Cos1A1}$ mutants: If $pka\text{-}RII^{Cos1A1}$, and thus RII overproduction, activates Hh signaling by regulating PKA-C1, PKA catalytic activity should be altered in $pka\text{-}RII^{Cos1A1}$ mutants. To address this question, PKA kinase assays were performed using extract from wild-type [Canton-S (CS)] or $cos2^{V1}$ $pka\text{-}RII^{Cos1A1}$ homozygous mutant embryos (Figure 5 and supplementary Figure 3 at http://www.genetics.org/supplemental/). Compared to wild type, the basal level of activity (no added cAMP) of PKA in $pka\text{-}RII^{Cos1A1}$ mutants is decreased. At the lowest level of cAMP tested, in $pka\text{-}RII^{Cos1A1}$ mutants, PKA activity is actually lower than in the presence of no added cAMP. This is in contrast to the wild-type situation where, even at the lowest level of cAMP, PKA activity is increased compared to the basal level of activity. At cAMP concentrations of 0, 0.005, and 0.05 μM, PKA activity in $pka\text{-}RII^{Cos1A1}$ mutants is decreased compared to wild type. At higher concentrations of cAMP (0.5 and 5 μM), PKA activity in $pka\text{-}RII^{Cos1A1}$ mutants was actually higher than that in wild type. At all concentrations tested, the activities of PKA in CS and $pka\text{-}RII^{Cos1A1}$ extracts were statistically different from each other on the basis of Student's *t*-test ($P < 0.05$; Figure 5).

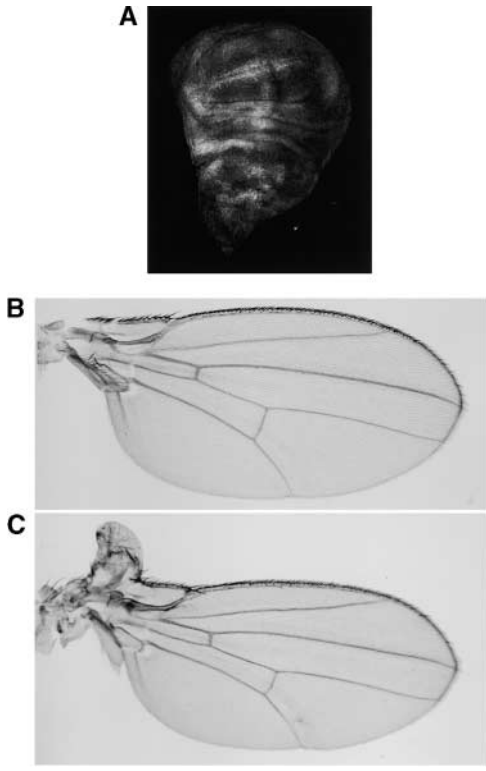
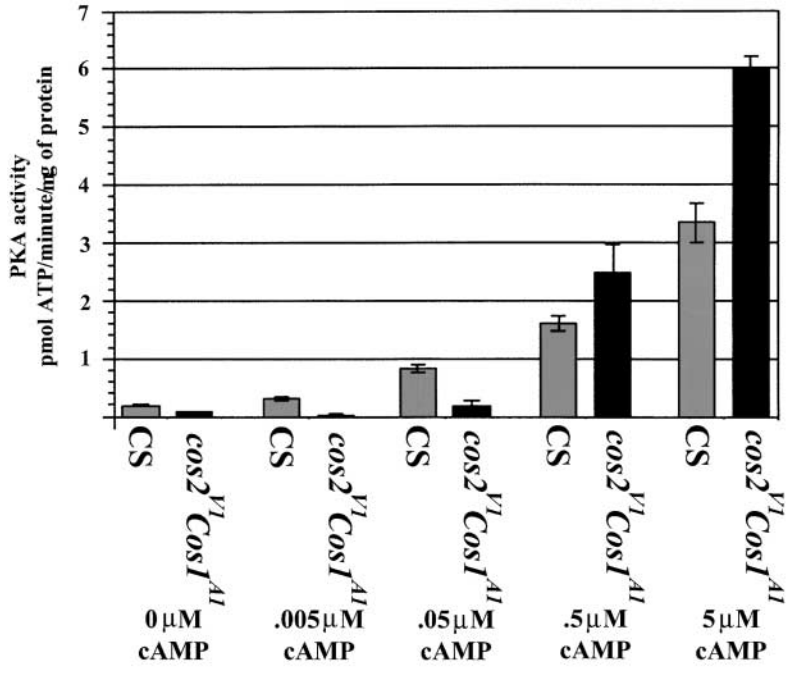


Figure 4.—Overexpression of RII is sufficient to cause *Cos1* phenotypes. *JW1 Gal4* was used to drive expression of RII using *EP(2)2162* in the developing wing. (A) The wing imaginal disc expression pattern of *JW1 Gal4* at 29° revealed using *UAS-GFP*. (B) A wing from an *EP(2)2277/JW1* male reared at 29°. *EP(2)2277* is a control EP element on chromosome arm 2R. (C) A wing from an *EP(2)2162/JW1* male reared at 29°. A duplication is present in the proximal costa region.

In conclusion, $pka\text{-}RII^{Cos1A1}$ results in decreased basal PKA catalytic activity but increased cAMP-stimulated PKA activity. The increase in cAMP-stimulated PKA activity is apparent only when high levels of cAMP are added, indicating that RII overproduction is likely to repress PKA catalytic activity *in vivo*. This supports the hypothesis that RII exerts its effects on Hh target gene transcription by inhibiting PKA-C1 activity.

Only certain cells are sensitive to *pka-RII* overexpression: *Cos1* was named due to the mutant phenotype of duplications limited to the costa region of the wing (Whittle 1973, 1974). Are only costa cells sensitive to the action of $pka\text{-}RII^{Cos1A1}$? Levels of full-length Ci (the activator form) and of *dpp* transcription were examined in $cos2^{V1}$ $pka\text{-}RII^{Cos1A1}/+$ mutant discs. In the absence of Hh, Ci is proteolytically cleaved to a 75-kD repressor form. Near the source of Hh, this proteolysis is inhibited and full-length Ci accumulates. This accumulation of full-length Ci can be monitored using 2A1, a monoclonal antibody that specifically recognizes full-length



Concentration cAMP (μM)	Genotype	Specific Activity	Standard Deviation
0	CS	0.193	0.019
0	*cos2 V1 Cos1 A1*	0.078	0.011
0.005	CS	0.314	0.045
0.005	*cos2 V1 Cos1 A1*	0.026	0.029
0.05	CS	0.823	0.068
0.05	*cos2 V1 Cos1 A1*	0.187	0.097
0.5	CS	1.601	0.135
0.5	*cos2 V1 Cos1 A1*	2.493	0.471
5	CS	3.349	0.335
5	*cos2 V1 Cos1 A1*	5.984	0.236

p 0 μM=.0008 p .005 μM=.0007 p .05 μM=.0007
p .5 μM=.0345 p 5μM=.0004

FIGURE 5.—PKA activity is affected in $Cos1^{A1}$ mutants. Kinase assays were performed using extract from wild type (CS) and *b pr* $cos2^{V1}$ *cn* $Cos1^{A1}$ homozygous mutant embryos. Activity is measured as picomoles of ATP per minute per microgram of protein. cAMP concentration varied from 0 to 5 μM. Values for CS are shaded and for *b pr* $cos2^{V1}$ *cn* $Cos1^{A1}$, solid. All reactions were performed in triplicate and standard deviations are indicated by error bars. Activity is the average of separate reactions performed in triplicate. The standard deviation is also given. For each concentration of cAMP, PKA activities in CS and *b pr* $cos2^{V1}$ *cn* $Cos1^{A1}$ extract were significantly different from each other using Student's *t*-test (*P* values range from <0.0007 to <0.0345).

Ci (AZA-BLANC *et al.* 1997). $cos2^{V1}$ $pka\text{-}RII^{Cos1A1}/+$; *dpp LacZ* wing discs were stained with 2A1 to monitor Ci. They were also labeled with anti-LacZ to monitor *dpp* transcription (Figure 6A). In $cos2^{V1}$ $pka\text{-}RII^{Cos1A1}/+$ discs, *dpp LacZ* is expressed in the costa region in addition to its normal expression just anterior to the A/P boundary. The costa region also has levels of full-length Ci comparable to, or even higher than, those seen at the A/P boundary where Hh signaling is active. No ectopic *hh* is detected in $pka\text{-}RII^{Cos1A1}$ mutants (data not shown). Therefore, *RII* overexpression can mimic the response of cells to Hh in a region of the disc where Hh is not present. In $cos2^{V1}$ $Cos1^{A1}/+$ discs, RII protein is expressed at equal levels throughout the entire disc (Figure 6A′ and data not shown), but ectopic high levels of full-length Ci and ectopic *dpp* transcription occur only in the region of the disc fated to form the costa of the adult wing (Figure 6, A′′ and A′′′).

To further investigate if the effects of RII are limited to the costa region or if all cells in the anterior compartment are competent to respond to RII, $pka\text{-}RII^{Cos1A1}$ clones and *JW1-Gal4-EP(2)2162* RII overproducing cells were generated throughout the wing imaginal disc (Figure 6, B–F). *dpp* transcription was assayed in these discs

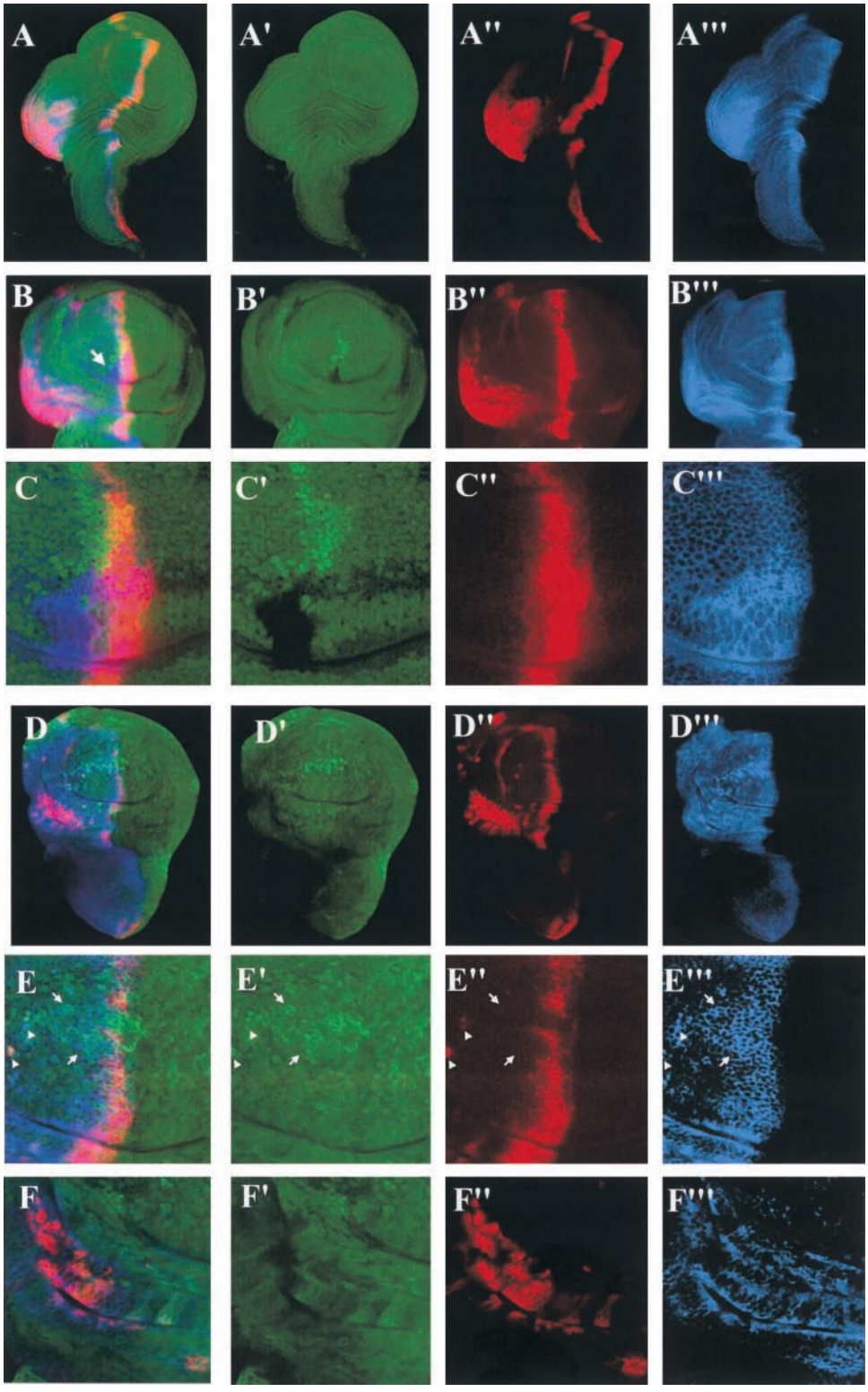


FIGURE 6.—Ci protein and *dpp* transcription in RII overexpressing cells. (A–A′′′) A third instar *b pr cos2*^{V1} *cn pka-RII*^{Cos1A1} */+; dpp-LacZ* wing imaginal disc. (B–B′′′) A third instar wing imaginal disc with a *pka-RII*^{Cos1A1} homozygous mutant clone just anterior to the A/P boundary (arrow). (C–C′′′) Close up of clone from B, revealing the detail of full-length Ci accumulation and *dpp-LacZ* expression. (D–D′′′) A third instar wing imaginal disc in which RII is overproduced using *JW1 Gal4* and *EP(2)2162*. (A′) Anti-RII staining reveals that RII levels are uniform throughout the disc. (A′′) Anti-LacZ staining visualizes *dpp-LacZ*. (A′′′) 2A1 antibody visualizes full-length Ci. Only in the presumptive duplication is Ci stabilized at levels comparable to or higher than those found at the A/P boundary. The high levels of *dpp LacZ* observed in the duplication (A′′) indicate that *dpp* is fully transcriptionally activated by Ci activator and not merely derepressed due to low levels of Ci75. (B′) The clone of *pka-RII*^{Cos1A1} homozygous mutant cells is marked by the lack of GFP expression. (B′′) Anti-LacZ staining visualizes *dpp-LacZ*. *dpp-LacZ* is present at the A/P boundary, but does not fill the clone, although full-length Ci is found throughout the clone (B′′′). (B′′′) 2A1 staining reveals full-length Ci. Full-length Ci accumulates throughout the *pka-RII*^{Cos1A1} homozygous mutant clone. (D′) Anti-RII staining reveals that *JW1 Gal4* drives *pka-RII* expression in patches of cells throughout the disc. (D′′) Anti-LacZ staining reveals the normal expression of *dpp-LacZ* at the A/P boundary and additional areas of ectopic expression. (D′′′) 2A1 staining reveals full-length Ci. Full-length Ci accumulates in anterior cells that overproduce RII. (E–E′′′) A close-up view of the A/P boundary of the wing pouch of the disc shown in D. Arrows indicate groups of cells that overproduce RII, have increased amounts of full-length Ci, but do not express *dpp-LacZ*. Arrowheads mark groups of cells that overproduce RII, have increased amounts of full-length Ci, and do express *dpp-LacZ*. (F–F′′′) A close-up view of the costa region of the disc shown in D. Many cells in the costa region ectopically express *dpp-LacZ*. A, B, C, D, E, and F are merged images. A′, D′, E′, and F′, anti-RII; B′ and C′, GFP; A′′, B′′, C′′, D′′, E′′, and F′′, anti-LacZ to visualize *dpp-LacZ*; A′′′, B′′′, C′′′, D′′′, E′′′, and F′′′, 2A1 antibody to visualize full-length Ci.

by staining for *dpp LacZ*. In *pka-RII*^{Cos1A1} homozygous mutant clones near the boundary, *dpp-LacZ* is not transcribed even though high levels of full-length Ci are present throughout the clone (Figure 6B and close-up view in 6C). However, cells in the costa region that are heterozygous for *pka-RII*^{Cos1A1} contain high levels of full-

length Ci and also transcribe *dpp* (Figure 6B). Absence of *dpp-LacZ* expression in *pka-RII*^{Cos1A1} homozygous mutant clones was observed in clones located in several regions of the anterior compartment (data not shown). When RII is overproduced using *JW1-Gal4* and *EP(2) 2162*, cells that contain high levels of RII contain high levels of full-length Ci. *JW1-Gal4* mediates RII overproduction in small groups of cells throughout the wing imaginal disc (Figure 4A and Figure 6, D′, E′, and F′). However, in the pouch region, only a few of the sporadic cells that overproduce RII and have high levels of full-length Ci also transcribe *dpp* (Figure 6E′′). In the costa region, many cells that overproduce RII and have high levels of full-length Ci transcribe *dpp* (Figure 6D′′).

In all situations in which RII is overproduced, only certain cells transcribe the Hh target gene *dpp-LacZ*. These responsive cells are found in more anterior regions of the disc. This argues for the existence of additional components of the Hh transduction machinery that are expressed or function only in limited regions of the wing imaginal disc.

DISCUSSION

Cos1 mutations strongly influence Hh signaling, but the identity of the gene has been mysterious for some time. Here we provide evidence in favor of PKA genes as the sites of *Cos1* mutations. Four alleles of *Cos1* were mapped to polytene chromosome location 30C while a fifth allele, *Cos1*^{A1}, was mapped to a different chromosome arm at polytene band 46E. We conclude that the two genes had previously been characterized as one because *Cos1*^{A1} was semiviable only *in trans* to the other *Cos1* alleles, and all alleles have the same genetic and phenotypic characteristics in combination with *cos2* alleles. We have identified the two genes as encoding subunits of PKA.

Two different map locations have previously been published for *Cos1*. Neither polytene band 30C nor 46E (this work) is consistent with the 50A1–50A2 map location for *Cos1*. *Cos1* was placed at 50A1–50A2 due to the discovery of a deficiency that deleted the region and resulted in wing duplications when *in trans* to a recessive allele of *cos1* (Lasko and Pardue 1988). It seems quite possible that a third gene was mapped that has genetic and phenotypic characteristics similar to the two *Cos1* genes that we have mapped. It was assumed in those studies that the *Cos1* phenotype results from haplo-insufficiency and that the *Cos1* phenotype is due to the deficiency and not to a second site mutation. For example, along with the deficiency, the chromosome may carry an allele of *cos2* that caused the observed phenotype.

The location of *Cos1*^{A1} is consistent with the Grau and Simpson (1987) map location, indicating that the *Cos1*^{A1} stock used in these experiments is the same stock used in their research. The map locations for our copies of the *Cos1*^{2} and *Cos1*^{3} mutations are not in agreement with their previously published map locations. Details of the genetic methods used were not presented in the original mapping article, so it is difficult to explain the discrepancy. One possibility is that in the many years that have passed since the original article there were mistakes in stock labeling. This is a distinct possibility since both *Cos1*^{2} and *Cos1*^{9} contain the same base-pair change and thus may have originally been the same stock. Both stocks are likely to carry the original *Cos1*^{9} allele as it was not previously mapped and the original *Cos1*^{2} allele was mapped to the opposite arm of chromosome 2.

***Cos1*^{2}, *Cos1*^{3}, *Cos1*^{8}, and *Cos1*^{9} are dominant-negative alleles of *pka-C1*:** We identified *pka-C1* as a candidate for the gene mutated in *Cos1*^{2}, *Cos1*^{3}, *Cos1*^{8}, and *Cos1*^{9}. We detected sequence changes at the DNA level at the *pka-C1* locus, and these changes translated to substitutions in the coding sequence. These point mutations could influence PKA activity in several possible ways. First, they could render PKA-C1 catalytically inactive and give dominant phenotypes due to haplo-insufficiency. Alternatively, the point mutations could destabilize the encoded protein to such a degree that the mutant protein would be degraded and thus function as a protein null. This scenario would also result in dominant phenotypes due to haplo-insufficiency. These haplo-insufficiency explanations are unlikely to be correct. *pka-C1* is a recessive negative regulator of Hh signaling and heterozygosity for *pka-C1* null alleles or for deficiencies that delete *pka-C1* does not result in any obvious phenotype. Second, the mutations could render PKA-C1 constitutively active. The unregulated activity could be responsible for the dominant wing duplication phenotypes. However, expression in the wing imaginal disc of a mutant PKA-C1 that cannot be regulated by cAMP interferes with the normal expression pattern of the Hh target gene *ptc*, indicating that constitutive PKA-C1 activity antagonizes Hh signal transduction (Jiang and Struhl 1995; Li *et al.* 1995).

We therefore favor a third possibility: that *pka-C1*$^{Cos1-2}$, *pka-C1*$^{Cos1-3}$, *pka-C1*$^{Cos1-8}$, and *pka-C1*$^{Cos1-9}$ encode dominant-negative (dn) versions of PKA-C1 that produce stable, full-length protein with reduced catalytic activity. There are precedents for the formation of dn *pka-C1* mutants in Drosophila. A previously described dominant mutation that causes wing duplications maps near *pka-C1*. Although the molecular nature of this mutation was not ascertained, it was assumed that the mutation was a *pka-C1* allele (Pan and Rubin 1995). Overproduction of a catalytically inactive mutant form of PKA-C1 in an otherwise wild-type background results in wing duplications, indicative of inappropriate activation of Hh signaling (Kiger and O'Shea 2001).

PKA-C1, like all other protein kinases, contains a catalytic core. Within the catalytic core of PKA-C1 and all kinases are defined subdomains that contain conserved amino acids and that play known roles in catalyzing the

phosphate transfer reaction. The amino acids affected in *pka-C1*$^{Cos1-2}$, *pka-C1*$^{Cos1-3}$, *pka-C1*$^{Cos1-8}$, and *pka-C1*$^{Cos1-9}$ mutants are part of the catalytic core and are likely to be required for PKA-C1 catalytic activity.

pka-C1^{Cos1-8} contains a substitution of D for G at aa 189 (all numbering of amino acids is taken from the protein sequence of Drosophila PKA-C1). This amino acid is the third amino acid in the DFG triad located in subdomain VII. This triad is conserved in essentially all serine/threonine and tyrosine kinases. In PKA, the triad has been implicated in binding the metal ion that coordinates the β- and γ-phosphoryl oxygens of the ATP used as a phosphate donor. Mutation of the D residue in the triad renders PKA inactive (Zoller *et al.* 1991). Therefore, it is likely that the substitution in *pka-C1*$^{Cos1-8}$ results in a catalytically inactive kinase.

pka-C1^{Cos1-3} contains a K substitution for E173. This amino acid lies in subdomain VI between D169 and N174, two residues implicated in ATP binding (Hanks *et al.* 1988). This region is implicated in substrate binding specificity as most serine/threonine kinases share the consensus 169-DLKPEN-174. Conversely, the sequence in most tyrosine kinases is DL(R/A)A(A/R)N (Hanks *et al.* 1988). The substitution in *pka-C1*$^{Cos1-3}$ may render PKA-C1 unable to recognize and phosphorylate serine and threonine residues. Inability to recognize appropriate substrates would render PKA-C1 catalytically inactive.

pka-C1^{Cos1-2} and *pka-C1*$^{Cos1-9}$ contain a substitution of K for E at aa 130 in the sequence 128-GGEMF-132. These amino acids are near the start of subdomain V, a subdomain that is not well conserved at the sequence level among protein kinases. However, this region may be structurally important. Crystal structures of kinase domains reveal two large lobes: an N-terminal lobe and a C-terminal lobe. The N-terminal lobe consists mainly of an antiparallel β-sheet and an α-helix. The C-terminal lobe is mainly helical but in PKA does contain four short β-strands. These two lobes are separated by a linker and form a pocket in which the substrate is phosphorylated. E130 is the first residue in the first α-helix in the C-terminal lobe. A substitution of a basic K for the acidic E could disrupt the secondary structure, change the orientation of the N and C lobes relative to each other, and thus influence the structure of the catalytic cleft.

The ability of a catalytically impaired kinase to function in a dn fashion can be explained in several ways. For example, a catalytically inactive kinase could still bind target proteins and thus actually compete with the functional kinases for substrate. An informative example of dn proteins comes from studies of growth factor receptors. Many growth factor receptors possess kinase activity and function as dimers. Production of a catalytically inactive subunit essentially "poisons" each dimer that it forms, leading to a dn phenotype. The inactive PKA holoenzyme consists of a tetramer of two R and two C subunits, but cAMP binding results in release of active C subunits that function as monomers. Therefore, catalytically active PKA-C1 is not part of a dimer and is not likely to be inactivated by virtue of association with a damaged subunit. However, the damaged subunit might interfere with release of an active catalytic subunit after cAMP binding. It is also possible that released catalytic PKA-C1, although not a dimer, might function as part of a larger multiprotein complex. In this case, a catalytically inactive PKA-C1 could still incorporate into complexes and render the entire complex ineffective.

***Cos1*^{A1} is a gain-of-function allele of RII:** *pka-RII*^{Cos1A1} was recovered in a screen using γ-rays as a mutagen. Unlike most chemical mutagens, which tend to cause point mutations, γ-rays tend to produce larger aberrations such as chromosome deficiencies or rearrangements. A change consistent with a 100-kb inversion was found in the 5′ UTR of *pka-RII* in *pka-RII*^{Cos1A1} mutants. No other changes in RII coding sequence were detected but higher levels of RII were found in *pka-RII*^{Cos1A1} mutant cells. On the basis of *cos2* clone results, this is not due to regulation of *pka-RII* by Hh signaling. In addition, *pka-RII*^{Cos1A1} clones located in the posterior compartment of the wing disc have high levels of RII but do not express Ci, the only transcription factor known to function in Hh signaling (data not shown). Taken together, the data indicate that the high levels of RII observed in the wing disc are unique to *pka-RII*^{Cos1A1} mutants. *pka-RII* overexpression is likely to cause the inappropriate activation of Hh target gene transcription observed in *pka-RII*^{Cos1A1} mutants. Previous work (Li *et al.* 1995) has shown that overexpression of a mutant RI that cannot bind cAMP can activate Hh target gene transcription. The data presented here are the first to show that the other regulatory subunit found in Drosophila, *pka-RII*, can influence Hh signaling and that overproduction of a wild-type R subunit of PKA is sufficient to activate Hh target gene transcription.

Antibody staining for RII in wing imaginal discs allowed us to observe that RII levels are increased in *pka-RII*^{Cos1A1} mutants but did not allow us to examine absolute levels or possible post-translational modifications. We therefore used Western analysis to examine RII protein levels in *pka-RII*^{Cos1A1} mutant embryos. RII protein exists in two forms due to differential phosphorylation of a consensus PKA phosphorylation site in RII (Foster *et al.* 1984). In Drosophila whole-fly extract, RII can be labeled by radiophosphate at low, but not high, levels of cAMP (Park *et al.* 2000). Increasing the cAMP concentration leads to the dissociation of R and C subunits, so the phosphorylation probably occurs when the RII subunit is part of a heterotetrameric holoenzyme (Rangel-Aldao and Rosen 1976). In *pka-RII*^{Cos1A1} mutants, the majority of the extra RII is the faster-migrating, nonphosphorylated form. This suggests that in *pka-RII*^{Cos1A1} mutants, RII is in great excess relative to C subunits and RII is not phosphorylated because it is not in holoen-

zymes. In this situation, essentially all PKA-C1 is predicted to be in a complex with RII.

Unlike the four alleles *pka-C1*$^{Cos1-2}$, *pka-C1*$^{Cos1-3}$, *pka-C1*$^{Cos1-8}$, and *pka-C1*$^{Cos1-9}$ that are likely to be antimorphic and function as *pka-C1*-dominant negatives, *pka-RII*^{Cos1A1} is likely to be a hypermorphic gain-of-function allele. Genetically, increased *pka-RII* function is predicted to mimic loss of *pka-C1* function. The wing duplications caused by Gal4-driven overexpression of *pka-RII* were, exactly as predicted, similar to the effects of *pka-RII*^{Cos1A1}. Overproduction of RII could influence PKA-C1 activity in two ways. First, RII could inhibit the catalytic activity of PKA by binding to the catalytic subunit. Second, R subunits can associate with A-kinase anchoring proteins (AKAPs) that localize PKA holoenzymes to specific subcellular locations (Scott and McCartney 1994). RII overproduction could mislocalize PKA-C1 within the cell via association with various AKAPs.

RII overproduction modulates PKA activity: The most likely way for overproduced RII to influence Hh signaling is by reducing PKA catalytic activity. The results of our PKA kinase assays are consistent with this hypothesis. In the heterotetrameric PKA holoenzyme, R subunits have two roles: inhibiting the catalytic activity of C subunits and protecting C subunits from degradation (Amieux *et al.* 1997; Brandon *et al.* 1998). Loss of R subunits results in increased basal activity of PKA due to the presence of free catalytically active C subunits, but in a decrease in cAMP-induced PKA activity because the C subunits are not protected from degradation. In *pka-RII*$^{EP(2)2162}$ mutants, RII levels are reduced by 95% and cAMP-induced PKA catalytic activity is reduced by almost 60%, indicating that C subunits have been degraded (Park *et al.* 2000). In *pka-RII*^{Cos1A1} mutants, high levels of RII should protect C subunits from degradation. At basal levels of cAMP, the stabilized pool of C subunits should remain associated with RIIs and the overall effect would be a decrease in basal PKA activity compared to wild type, which is precisely what we observed.

With the addition of cAMP, RII subunits should release C subunits and PKA activity should increase. At high levels of cAMP, essentially all C subunits should be released from RII-mediated inhibition. In this situation, mutants should contain higher levels of PKA activity than wild type because the high levels of RII have protected C subunits from degradation, resulting in an overall increase in the amount of C subunits. For the most part, our measurements of PKA activity in *pka-RII*^{Cos1A1} compared to wild type were exactly as we predicted: basal PKA activity was decreased in *pka-RII*^{Cos1A1} mutants compared to wild type while cAMP-induced PKA activity was vastly higher in *pka-RII*^{Cos1A1} mutants than in wild type. However, at the lowest level of cAMP tested, a slight reduction in PKA activity from the basal level was observed for *pka-RII*^{Cos1A1} mutants. This contrasts to the wild-type situation, where any added cAMP results in PKA activity higher than basal levels. One possible explanation for the slight decrease of PKA activity in the mutant could be that the initial release of a small amount of C subunits triggers the degradation reaction. At higher levels of cAMP, this effect is masked because more C subunits are released than can be immediately processed by the degradation machinery. Although RII overexpression results in higher levels of cAMP-induced PKA activity compared to wild type, the basal level of PKA activity is lower in the mutant than in wild type.

In the future, it will be interesting to examine the roles of wild-type R subunits in Hh signal transduction. If *pka-RII* normally plays a role in transducing the Hh signal, *pka-RII* mutants should possess Hh phenotypes. The only reported *pka-RII* mutant, *pka-RII*$^{EP(2)2162}$, reduces adult RII levels by >95% and is homozygous viable with no phenotypes indicative of aberrant Hh signaling (Park *et al.* 2000). RI and RII may function redundantly. Another possibility is that residual levels of RII may be sufficient for normal regulation of Hh target gene transcription. The roles of R subunits in transducing the Hh signal may be revealed only by the creation of the double mutant carrying null alleles of RI and RII.

The wing duplication phenotype of *pka-RII*^{Cos1A1} indicates that Hh target gene transcription has been inappropriately activated in response to *pka-RII* overexpression in the anterior compartment of the wing imaginal disc. Although RII protein levels are uniform throughout *pka-RII*^{Cos1A1} heterozygous wing discs, Ci is stabilized and *dpp* is transcribed only in the presumptive duplication. Not every cell in the presumptive duplication that contains high levels of full-length Ci transcribes *dpp*. The high levels of full-length Ci should indicate that Ci repressor levels are low, so *dpp*, at the very least, should be derepressed. This sensitivity of only certain cells to high levels of full-length Ci resulting from RII overexpression was also observed when very high levels of RII were generated in *pka-RII*^{Cos1A1} homozygous clones or by GAL4-UAS-mediated expression. In general, anteriormost cells are the most sensitive to *pka-RII* overexpression. One possible explanation is that more medial cells contain higher levels of cAMP and thus higher levels of RII are required to suppress PKA-C1 activity.

A similar situation is observed in clones mutant for a component of the SCF ubiquitin ligase complex, *Roc1a*. In *Roc1a* mutant clones, *dpp* is transcribed only in the most anterior clones even though high levels of full-length Ci are detected in all anterior clones. This is surprising because the appearance of full-length Ci in these cells must correspond to a decrease in Ci75 repressor levels. In the absence of Ci75, *dpp* should be transcribed because it is derepressed. One possible interpretation is that in medial cells less full-length Ci is stabilized in response to *pka-RII* overexpression or loss of *Roc1a*. Because full-length Ci forms at the expense of Ci75, small reductions in full-length Ci levels translate

into small increases in Ci75 levels. This small increase in Ci75 could provide enough repressor activity to repress *dpp*. Immunofluorescense may not be sensitive enough to detect subtle differences in full-length Ci levels between medial and lateral cells.

Our studies of *Costal1*, together with previous work by others, provide a powerful genetic link between the kinesin-related molecule Cos2 and PKA. The genetic interaction may be due to the sensitive balance between Ci, which is modified by PKA, and Cos2, or to additional activities that more directly link PKA to Cos2. In imaginal disc development precisely controlled Hh signal transducer activities are crucial for pattern formation, building the perfect forms of wings and legs.

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