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Proteolysis of Cubitus interruptus in *Drosophila* requires phosphorylation by Protein Kinase A

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SUMMARY

The Hedgehog signal transduction pathway is involved in diverse patterning events in many organisms. In *Drosophila*, Hedgehog signaling regulates transcription of target genes by modifying the activity of the DNA-binding protein Cubitus interruptus (Ci). Hedgehog signaling inhibits proteolytic cleavage of full-length Ci (Ci-155) to Ci-75, a form that represses some target genes, and also converts the full-length form to a potent transcriptional activator. Reduction of protein kinase A (PKA) activity also leads to accumulation of full-length Ci and to ectopic expression of Hedgehog target genes, prompting the hypothesis that PKA might normally promote cleavage to Ci-75 by directly phosphorylating Ci-155. Here we show that a mutant form of Ci lacking five potential PKA phosphorylation sites (Ci5m) is not detectably cleaved to Ci-75 in *Drosophila* embryos. Moreover, changes in PKA activity dramatically altered levels of full-length wild-type Ci in embryos and imaginal discs, but did not significantly alter full-length Ci5m levels. We corroborate these results by showing that Ci5m is more active than wild-type Ci at inducing ectopic transcription of the Hh target gene *wingless* in embryos and that inhibition of PKA enhances induction of *wingless* by wild-type Ci but not by Ci5m. We therefore propose that PKA phosphorylation of Ci is required for the proteolysis of Ci-155 to Ci-75 in vivo. We also show that the activity of Ci5m remains Hedgehog responsive if expressed at low levels, providing further evidence that the full-length form of Ci undergoes a Hedgehog-dependent activation step.

Key words: Hedgehog, Cubitus interruptus, Protein kinase A, Proteolysis, *Drosophila*

INTRODUCTION

The Hedgehog (Hh) class of secreted signaling molecules is important in a number of patterning events in the development of organisms from fly to man (reviewed in Hammerschmidt et al., 1997; Ingham, 1998). In *Drosophila* embryos, Hh is expressed in the posterior cells of each segment and maintains the production of *wingless (wg)*, another secreted signaling molecule, in neighboring anterior cells. In wing imaginal discs, Hh is expressed in posterior (P) compartment cells, and leads to production of *decapentaplegic (dpp)* in a stripe of anterior (A) compartment cells close to the A/P border. Dpp, a TGF-β family member, then acts as a morphogen that controls A/P polarity and growth of the disc. Studies of these processes in flies have been instrumental in identifying components in the Hh pathway and in elucidating the mechanism by which these components regulate target gene expression.

The current model for Hh signal transduction is as follows. In the absence of Hh signaling, the transmembrane protein Patched (Ptc) inhibits Smoothened (Smo), another transmembrane protein, from activating the pathway intracellularly (Ingham, 1998). When Hh binds Ptc, this inhibition is relieved, resulting in several changes in the cell. A large complex, containing genetically identified Hh pathway components Costal2 (Cos2) and Fused (Fu), is released from microtubules (Robbins et al., 1997; Sisson et al., 1997) and both these proteins become more highly phosphorylated (Thérond et al., 1996; Robbins et al., 1997). Cubitus interruptus (Ci), the *Drosophila* homologue of the vertebrate Gli family of zinc-finger DNA-binding proteins (Orenic et al., 1990), is another component of this complex. In cells that do not receive the Hh signal, Ci is partially proteolysed to yield a 75 kDa form ('Ci-75') consisting of the N-terminal half of the protein (Aza-Blanc et al., 1997). The 75 kDa form of Ci has been shown to act as a repressor on some, but not all, Hh-responsive promoters in wing imaginal discs (Aza-Blanc et al., 1997; Méthot and Basler, 1999). In cells exposed to Hh, the proteolytic cleavage of Ci is inhibited, thereby increasing the steady-state level of full-length Ci protein ('Ci-155') (Motzny and Holmgren, 1995; Slusarski et al., 1995; Aza-Blanc et al., 1997). Hh also acts to change Ci-155 into a more potent transcriptional activator (Ohlmeyer and Kalderon, 1998; Méthot and Basler, 1999). The mechanism by which Hh enhances the transcriptional activity of Ci remains largely unknown, but it has been shown that Su(fu), a pathway component that binds to Ci (Monnier et al., 1998), inhibits this process in a dose-dependent manner, and that the serine/threonine kinase Fu counteracts the action of Su(fu) in response to Hh signalling (Ohlmeyer and Kalderon, 1998). The result of both actions of Hh, inhibition of proteolysis and

activation of Ci-155, is that at physiological levels of Ci, target gene expression is Hh dependent. If Ci expression is increased artificially above physiological levels, however, target gene expression can become independent of Hh, leading to ectopic transcription of *wg* in embryos and of *dpp* and *ptc* in wing imaginal discs (Domínguez et al., 1996; Alexandre et al., 1996; Hepker et al., 1997).

Mutations in several genes, including *cos2*, the gene encoding cAMP-dependent protein kinase (PKA), and *supernumerary limbs (slimb)*, cause cell autonomous accumulation of Ci-155 and ectopic expression of Hh target genes in anterior compartment cells of imaginal discs (Johnson et al., 1995; Sisson et al., 1997; Jiang and Struhl, 1998). For *PKA* and *slimb* mutations, the accumulation of Ci-155 was accompanied by a drastic reduction in Ci-75 levels, implying that the primary effect of these mutations was to block cleavage of Ci-155 to Ci-75 (Jiang and Struhl, 1998). In embryos, reduction of PKA activity also leads to inhibition of Ci cleavage and results in modest anterior expansions of *wg* expression (Ohlmeyer and Kalderon, 1997, 1998). Furthermore, in imaginal discs, increasing PKA activity can reduce Hh target gene expression at the A/P border (Li et al., 1995).

Here we have examined the mechanism by which PKA promotes processing of Ci. Specifically, we altered potential PKA phosphorylation sites in Ci and introduced transgenes encoding these altered proteins into flies to test the idea that PKA promotes Ci cleavage by directly phosphorylating it, as has been suggested by experiments in cultured *Drosophila* cells (Chen et al., 1998). We found that a Ci variant lacking five potential PKA sites (Ci5m) was not proteolytically cleaved. Furthermore, full-length Ci5m levels and the transcriptional activity of Ci5m were essentially unaffected when PKA activity was reduced in embryos or increased in wing discs. We conclude that the PKA sites altered in Ci5m are essential for PKA to promote proteolysis of Ci-155 to Ci-75.

MATERIALS AND METHODS

Mutagenesis and cloning

Mutations were introduced into the *ci* cDNA using M13 single-stranded DNA containing the appropriate *ci* cDNA fragment using standard methods and the following oligonucleotides:

1 GTTTCGTCGCGATGCGCAAAATTCCAGTG
2 CAAAGTCGTCGGGCCGCTCAATCATCCCA
3 GCTCACGGCGCGCTGCTCAAATGTCAAATG
a TATGCGTCGGCATGCCGGTGAAAAGCCAC
b AAAACGTTACGCGGACCCAAGCTCTTTGA
A ACAGCGGAAAGATGCTGAGTATCACAATT
B TGCACGACGCCAAGCTGATCCAGTGCCAA

The underlined bases denote those that have been changed from the wild-type cDNA. Double-stranded DNA fragments containing mutant sites were cloned into pGEM7Zci (Hepker et al., 1997). HA and TBP tags were joined to the N terminus of Ci by adding DNA encoding amino acids MYPYDVPDYANSSGT or MDQNNSLPPYANSSGT (Brou et al., 1993), respectively, before the normal start codon of Ci (epitope tag is underlined). Tagged and untagged Ci constructs were then subcloned into pUAST (Brand and Perrimon, 1993) for P-element-mediated germline transformation. Sequences originating from M13 clones or PCR were sequenced in their entirety. pUAST-Ci constructs were injected into embryos by standard methods and autosomal transgenic lines kept. Second chromosome insertions were recombined with second chromosome transgenes *UAS-R** and *UAS-mC** (Li et al., 1995).

Crosses

To obtain embryos lacking endogenous *ci* and expressing transgenic *ci*, (a) *UAS-Ci* ± *UAS-R**/ (*P[y+]CyO*); *Df(4)M62f* / (ey^D or +) or (b) (*UAS-R** or +) / *P[y+]CyO*; *UAS-Ci*/ +; *Df(4)M62f* / + males were crossed to *RG1* / +; *Df(4)M62f* / ey^D females (*RG1* is *prdGAL4* (Yoffe et al., 1995) and *Df(4)M62f* deletes the *ci* gene). To remove maternal Su(fu), *RG1* $Su(fu)^{LP}$/ $Su(fu)^{LP}$; *Df(4)M62f* / ey^D females were used. For embryo extracts, crosses were made between (*UAS-Ci* ± *UAS-R**) females and *E22C* (Ohlmeyer and Kalderon, 1997) males. For imaginal discs, crosses were made between (*UAS-R**, *UAS-mC** or +); *UAS-Ci* males and *71B* (Brand and Perrimon, 1993) females.

Preparation of samples for staining or immunoprecipitation

Embryos were collected for 3 hours on apple juice plates at room temperature then incubated for 5 hours at 29°C to obtain stage 10-13 embryos. Dechorionated embryos were fixed in formaldehyde by standard methods and stored in methanol at 4°C prior to antibody staining and RNA in situ hybridization. For extracts, dechorionated embryos were equilibrated in cold RIPA/glycerol buffer (150 mM NaCl, 1% NP40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 8, 1 mM DTT, 10% glycerol, 1 mM EDTA, and complete protease inhibitors (Boehringer Mannheim)), homogenized, centrifuged 15 minutes at 11,000 revs/minute at 4°C, and the supernatant frozen at –80°C. For imaginal disc staining, larvae were incubated for 3 days at 29°C before dissecting third instar larvae in cold PBS (1 hour), fixation in 1.5% formaldehyde at room temperature (1 hour) and storage in methanol at 4°C.

Antibody staining and in situ hybridization

Digoxigenin-labeled RNA probes synthesized using T7 RNA polymerase from *Pst*I-cut wgPx4 (Ohlmeyer and Kalderon, 1997) and *Bam*HI-cut pGEM7Zci (Hepker et al., 1997) were used for in situ hybridization to *wg* and *ci* RNA, respectively. Staining for full-length Ci protein was carried out by standard methods with rat monoclonal antibody 2A1 (Motzny and Holmgren, 1995) diluted 1:1 in 5% BSA, binding overnight at 4°C followed by visualization with the Vector ABC Elite kit (Vectastain).

Immunoprecipitation

Embryo extracts (600 μg protein) were combined with 1 μl anti-HA monoclonal antibody (12CA5) ascites for 30 minutes on ice. After addition of 50 μl 1:1 protein G Sepharose:RIPA slurry, samples were incubated 2-3 hours at 4°C with rocking. Beads were washed 3 times with 400 μl RIPA buffer and then 45 μl sample buffer were added. Samples were run on 8% SDS-polyacrylamide gels, transferred to nitrocellulose and probed with antibody raised to the zinc finger region of Ci at a dilution of 1:1000. Proteins were visualized using ECL (Amersham).

PKA assays

Immunoprecipitated samples were washed with RIPA, then twice with PKA assay buffer (50 mM MOPS, pH 7.0, 10 mM $MgCl_2$, 0.25 mg/ml BSA). Reactions were carried out in 30 μl PKA assay buffer with 2.5 U PKA catalytic subunit (Sigma) and 25 μM ATP (with 1 μCi [γ-^{32}P]ATP) for 20 minutes at 30°C. Reactions were stopped on ice and 10 μl 4× sample buffer added. Samples were run on 6% SDS-polyacrylamide gels, transferred to nitrocellulose and exposed to X-ray film.

RESULTS

The Ci protein sequence includes five optimal PKA phosphorylation sites (RRX(S/T); labeled **1**, **2**, **3**, **a** and **B** in

Fig. 1). Sequences lacking one of the consensus arginine residues are much poorer substrates for PKA but are sometimes phosphorylated in vivo (Walsh and Van Patten, 1994) and can be found in over 20 locations in Ci. We made site-directed mutants affecting all five consensus PKA sites of Ci and the two sites that contained only a conservative (K for R) substitution in the consensus (Fig. 1). In each case, the potential phosphoacceptor site was changed to alanine. We made the mutations in three separate groups, in order to try to make a form of Ci that was functional but potentially resistant to the effects of PKA. Ci3m contains changes to sites **1**, **2** and **3** (S838, S855, S856, S891, and S892; Fig. 1); Ci5m additionally contains changes to sites **A** and **B** (S962 and T1006); Ci7m contains the same changes as Ci5m plus sites **a** and **b** (T512 and T589) which fall in the DNA-binding domain of Ci. Constructs encoding untagged Ci, and Ci N-terminally tagged with epitopes from hemagglutinin (HA) or TATA-binding protein (TBP) were cloned downstream of five copies of a GAL4-responsive upstream activating sequence (UAS; Brand and Perrimon, 1993) and introduced into flies.

Ci lacking five potential PKA phosphorylation sites is not detectably proteolysed

In order to examine proteolysis directly, we made extracts of stage 10-13 embryos expressing wild-type or mutant HA-tagged Ci under the control of an 'enhancer trap' line, *E22C*, that expresses GAL4 ubiquitously in embryos. We immunoprecipitated extracts with anti-HA antibody and analyzed the antibody-bound proteins by probing the resulting western blot with a polyclonal antibody to the Ci zinc-finger domain, which detects both forms of Ci (Ohlmeyer and Kalderon, 1998). In embryos expressing HA-tagged wild-type Ci, a small, but readily detectable fraction of Ci was found in the 75 kDa form (Fig. 2, lanes 2 and 4). The ratio of Ci-155 (which migrates at ~180 kDa) to Ci-75 was similar to that obtained with endogenous protein in embryo extracts (Aza-Blanc et al., 1997; Ohlmeyer and Kalderon, 1998). Cleavage of endogenous Ci-155 to Ci-75 is blocked in embryos when PKA is inhibited by expressing R*, a mutant PKA regulatory subunit that binds constitutively to the catalytic subunit (Li et al., 1995; Ohlmeyer and Kalderon, 1997). Similarly, in

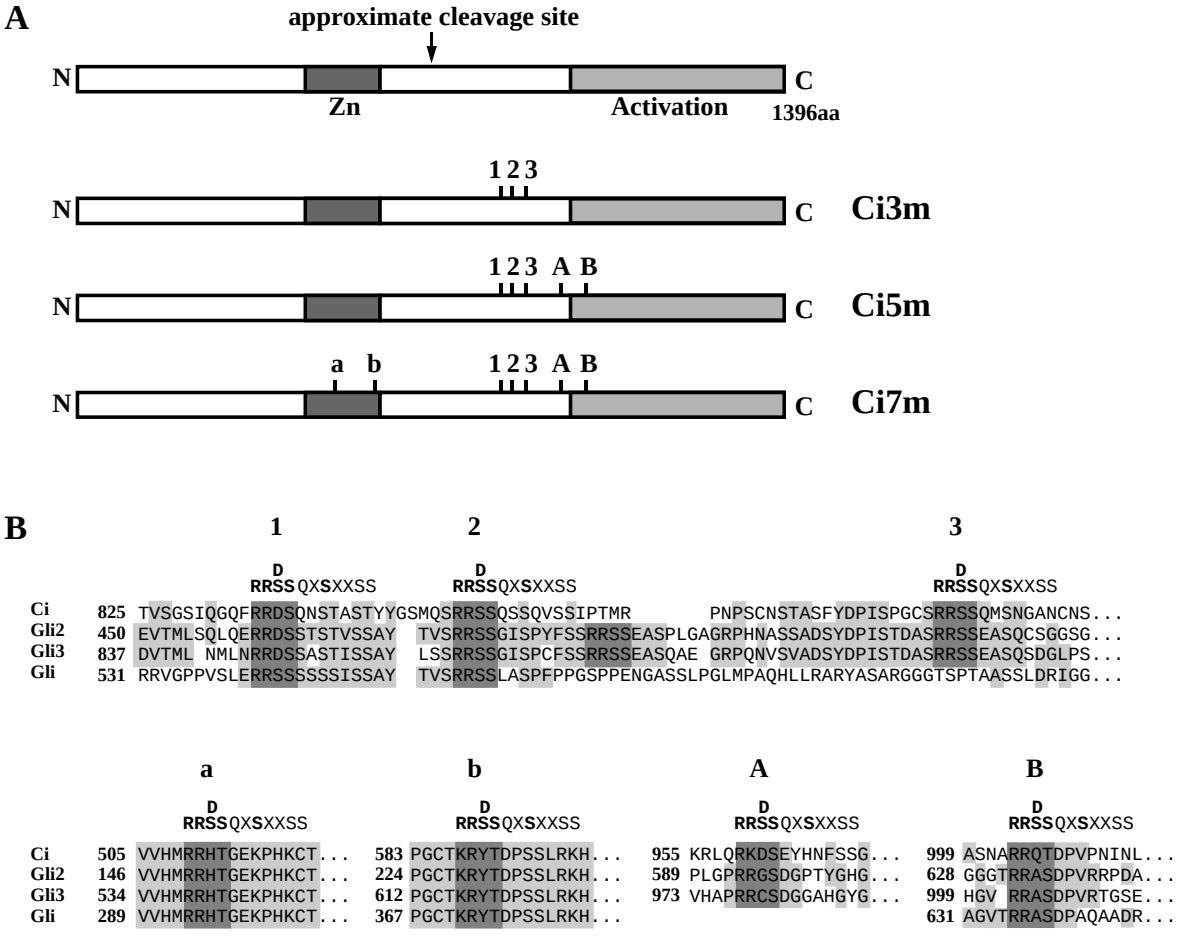

Fig. 1. (A) Schematic of Cubitus interruptus protein. The relative positions of the zinc-finger DNA-binding domain (dark shading), transcriptional activation domain (light shading) (Alexandre et al., 1996) and approximate cleavage site for Ci-75 generation (arrow) are indicated. Potential PKA sites altered in this study are labeled **a**, **b**, **1**, **2**, **3**, **A** and **B**. Ci3m, Ci5m and Ci7m contain alterations to three, five and seven sites, respectively, as indicated. (B) Alignment of sequences around the Ci PKA sites with Gli, Gli2 and Gli3 sequences from the corresponding regions (Orenic et al., 1990; Ruppert et al., 1990). The dark shading corresponds to each consensus PKA site; light shading indicates identity or similarity between two or more family members in other regions. A consensus for Ci sites **1**, **2**, and **3** (**RR**(**D**/**S**)QX**S**XXSS) is aligned above each PKA site; bold lettering indicates residues that are absolutely conserved among these three sites in Ci, Gli2, and Gli3; X corresponds to any amino acid.

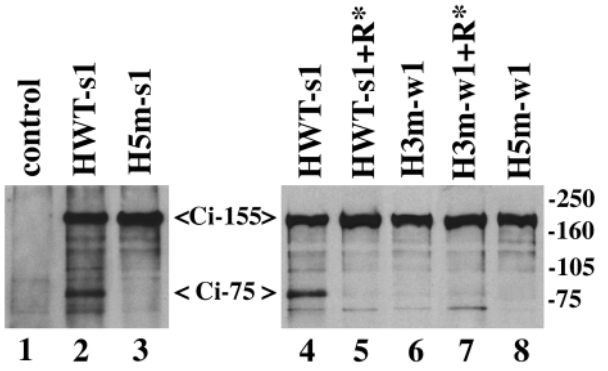

Fig. 2. Proteolysis of Ci-155 to Ci-75 is inhibited when PKA sites are altered. Western blots were probed with antibody to the zinc finger region of Ci following immunoprecipitations with anti-HA antibody. Immunoprecipitations were carried out on extracts of stage 10-13 embryos expressing no transgene (lane 1) or HA-tagged Ci (lanes 2-8) under the control of *E22C*. The particular wild-type Ci (HWT-s1), Ci3m (H3m-w1), and Ci5m (H5m-w1 and H5m-s1) lines used are shown above each lane. In lanes 5 and 7, embryos also expressed the PKA inhibitor R*. Molecular weights in kDa are indicated on the right. A very small amount of protein is detected at ~75 kDa in some Ci5m (lane 8) and Ci3m (lane 6) samples, but is not thought to be a product of specific cleavage since it is not always present in Ci5m samples (lane 3) and is not eliminated by co-expression of R* (lane 7).

embryos expressing HA-tagged wild-type Ci and R*, no Ci-75 was detected (Fig. 2, lane 5). In embryos expressing HA-tagged Ci5m or Ci3m, no 75 kDa form was detected even though the levels of the 155 kDa form were at least as high as in the wild-type samples (Fig. 2, lanes 3, 6, and 8). Thus, loss of three or five potential PKA phosphorylation sites in Ci blocked proteolysis, within the sensitivity of this assay.

Ci can be phosphorylated by PKA in vitro

To test whether PKA can directly phosphorylate Ci in vitro, we immunoprecipitated HA-tagged wild-type Ci and incubated with purified PKA catalytic subunit and radiolabeled ATP. A labeled protein was detected at ~180 kDa (Fig. 3A, lane 2), at the same position as Ci-155 detected with antibody to Ci. Phosphorylation of this protein was inhibited by the PKA-specific peptide inhibitor PKI (5-24) (Cheng et al., 1986; Fig. 3A, lane 3) and required the addition of PKA (Fig. 3A, lane 4); the observed phosphorylation is therefore unlikely to be due to a contaminating kinase in the immunoprecipitate. Ci5m was phosphorylated by PKA but to a lesser extent than wild-type Ci (Fig. 3B, lanes 5 and 6), suggesting that at least some of the sites corresponding to those altered in Ci5m can be phosphorylated by PKA.

Levels of full-length wild-type Ci and Ci3m, but not Ci5m, are reduced by excess PKA in wing imaginal discs

In wing imaginal discs, Hh expressed in the posterior (P) compartment induces high levels and activity of Ci-155 in anterior (A) cells at the A/P border, resulting in *dpp* expression in a stripe along the A/P border and uniform growth of the disc on either side. Because PKA hyperactivity can oppose Hh signaling at the A/P border (Li et al., 1995), we wanted to test if it could also affect proteolysis of Ci-155 encoded by wild-type or mutant *ci* transgenes. Expression of wild-type and mutant *ci* transgenes using the GAL4 line *71B* produced high levels of Ci-155 (detected by an antibody specific to this form of Ci) in A and P cells of the wing blade region and caused expansions of the discs in this region (Fig. 4). For the wild-type *ci* transgene, Ci-155 levels and expansions appeared to be greatest in the posterior compartment (Fig. 4C), whereas Ci3m and Ci5m appeared to affect A and P compartments equally (Fig. 4E,G). This difference is consistent with the idea that Ci3m and Ci5m are resistant to cleavage in all cells, whereas wild-type Ci-155 is efficiently converted to Ci-75 in A cells distant from the A/P border, where there is no Hh signalling (Méthot and Basler, 1999).

Increasing PKA activity by expression of a constitutively active catalytic subunit mC* (Li et al., 1995) in otherwise wild-type discs reduced Ci-155 levels in the wing-blade region of the A/P border and caused a narrowing of the disc in this region, indicative of reduced Ci activity (Fig. 4B). When PKA activity was increased in discs ectopically expressing wild-type Ci or Ci3m transgenes, the levels of full-length Ci in the posterior compartment were clearly reduced and the discs narrowed (Fig. 4D,H). In contrast, increased PKA activity neither reduced the levels of full-length Ci5m in the posterior compartment nor did it affect the size of discs expressing Ci5m in the wing blade region (Fig. 4F). Thus, our data suggest that abnormally high levels of PKA can promote the cleavage of wild-type Ci and Ci3m, but not Ci5m.

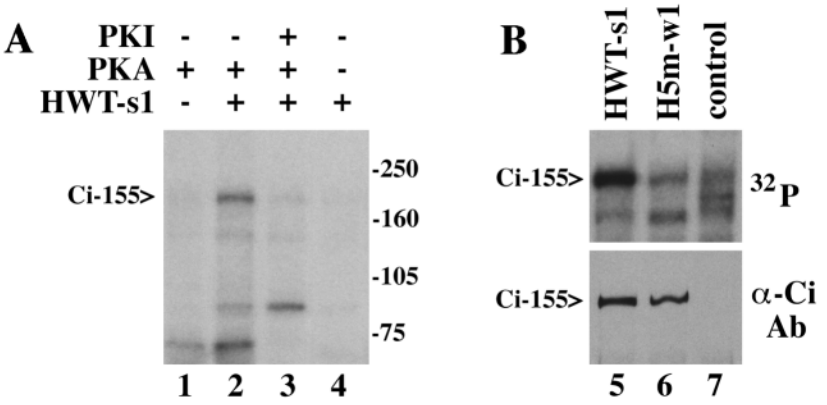

Fig. 3. Ci is phosphorylated by PKA in vitro. (A) Extracts of embryos expressing only endogenous Ci (lane 1) or the HA-tagged wild-type *ci* transgene HWT-s1 under the control of *E22C* (lanes 2-4) were immunoprecipitated with antibody to HA, incubated with [γ-^{32}P]ATP, PKA catalytic subunit (lanes 2 and 3) and the PKA inhibitor peptide PKI (5-24) (Cheng et al., 1986) at 1 μM (lane 3), prior to transfer onto nitrocellulose and autoradiography. (B) HA immunoprecipitates from extracts of embryos expressing only endogenous Ci protein (lane 7), wild-type Ci (HWT-s1; lane 5) and Ci5m (H5m-w1; lane 6) were incubated with PKA and [γ-^{32}P]ATP, prior to blotting and autoradiography (top panel). The same filter was probed with antibody to the Ci zinc-finger region (lower panel). Phosphorimager analysis showed that about 4.3 times as much ^{32}P was incorporated into HA-tagged wild-type Ci as Ci5m. In other experiments, the full-length Ci5m band was clearly distinct from background labeling seen in the control (lane 7).

Ci5m has greater specific activity than wild-type Ci in the absence of Hh signalling

It is known that an excess of wild-type Ci can activate Hh target gene expression even in cells where Hh is not signaling (Alexandre et al., 1996; Domínguez et al., 1996; Hepker et al., 1997). If Ci5m cannot be inactivated by proteolytic cleavage, the amount of *ci* RNA required to induce ectopic Hh target gene expression should be lower for Ci5m than for wild-type Ci. We tested this prediction first by crossing all of our autosomal homozygous viable transgenic *ci* lines to different homozygous GAL4-expressing lines and assaying embryonic or pupal lethality of progeny, as appropriate. In this way, we presumably included a similar spectrum of expression levels for each type of transgene.

In crosses to *E22C*, which expresses GAL4 ubiquitously in embryos, offspring of only 13% of untagged wild-type Ci lines died before hatching, whereas 48% of untagged Ci5m lines caused embryonic lethality (Table 1). The differences between wild-type and Ci5m were even more pronounced in crosses to *ptc*GAL4. This may be because Hh signaling positively regulates the *ptc* promoter, which directs expression in cells that normally express Ci. Expression of transgenic Ci in cells where Ci is not normally expressed (using *en*GAL4), also led to differences in embryonic phenotypes between wild-type (15% embryonic lethality) and Ci5m (36% embryonic lethality). Finally expression of Ci in wing imaginal discs (with GAL4 line *71B*) caused larval or pupal lethality for 31% of wild-type Ci lines and 71% of Ci5m lines. In all these assays, the same phenotypes and relative strengths of wild-type and Ci5m lines were seen when looking at HA- or TBP-tagged *ci* transgenes. Ci3m lines were most similar overall to Ci5m but were a little weaker in all assays (Table 1). Thus, Ci5m and, to a lesser extent, Ci3m exhibited greater activity than ectopically expressed wild-type *ci* transgenes. Ci7m lines rarely induced lethality in these assays and were also unable to support any *wg* expression in the assays described below (data not shown), indicating that the two PKA site alterations in the zinc-finger domain had drastically impaired a normal activity of the protein.

Table 1. Alteration of PKA sites enhances the activity of Ci

	Untagged	HA-tagged	TBP-tagged	Sum
(a) Percentage of lines giving embryonic lethality‡ with *E22C*				
WT	13 (2/15)*	6 (1/17)	0 (0/30)	5 (3/62)
3m	46 (6/13)	25 (3/12)	-	36 (9/25)
5m	48 (13/27)	44 (11/25)	38 (9/24)	43 (33/76)
(b) Percentage of lines giving embryonic lethality‡ with *ptc*GAL4				
WT	15 (2/13)	12 (2/17)	3 (1/30)	8 (5/60)
3m	69 (9/13)	55 (6/11)	-	63 (15/24)
5m	86 (24/28)	88 (23/26)	92 (22/24)	88 (69/78)
(c) Percentage of lines giving embryonic lethality‡ with *en*GAL4				
WT	15 (2/13)	12 (2/17)	13 (4/30)	13 (8/60)
3m	31 (4/13)	17§ (1/6)	-	26 (5/19)
5m	36 (10/28)	65 (17/26)	36 (8/22)	46 (35/76)
(d) Percentage of lines giving larval or pupal lethality with *71B*				
WT	31 (4/13)	6 (1/17)	37 (11/30)	27 (16/60)
3m	62 (8/13)	58 (7/12)	-	60 (15/25)
5m	71 (20/28)	77 (20/26)	79 (19/24)	76 (59/76)

‡No hatching or low hatching with early lethality for escaper larvae.
*(number of embryonic lethal lines/total lines tested).
§n=6; in all other cases $n \geq 11$.

Ci5m is more active than wild-type Ci in inducing ectopic Hh target gene expression

In each trunk segment of the ventral embryonic ectoderm of stage 9-12 wild-type embryos, *ci* is expressed in a wide band of A cells (Orenic et al., 1990). Hh is expressed in a complementary pattern in P cells and induces *wg* expression in a single row of A cells across the parasegmental (P to A) boundary. We used *prd*GAL4 to express *ci* transgenes in alternating segments of embryos lacking endogenous *ci* gene products and determined the induced pattern of *wg* expression in order to compare the activities of wild-type and mutant *ci* transgenes. Embryos lacking endogenous *ci* were readily identified by the absence of *wg* stripes from the seven odd-numbered parasegments. Each *prd*GAL4-induced stripe of *ci* expression spans the parasegment border, including all the P cells and about one-third to one-half of the immediately anterior A cells of stage 11 embryos (Yoffe et al., 1995; Fig. 5C schematic). All these cells are competent to express *wg* in the presence of excess Ci (Alexandre et al., 1996).

Nine out of ten wild-type *ci* lines supported single-cell-wide stripes of *wg* RNA in alternating segments of embryos lacking endogenous *ci* (Figs 5D,F, 7A; data not shown). *ci* RNA levels in these lines were assayed by in situ hybridization and estimated to range from less than endogenous levels to at least 3- to 4-fold higher (Fig. 5C,E; data not shown). One wild-type line, which was among the 8% that induced embryonic lethality in crosses to *ptc*GAL4, induced wide, anteriorly expanded, *wg* stripes in the absence of endogenous Ci (data not shown), indicative of Hh-independent *wg* induction. We therefore estimate that fewer than 10% of all our wild-type Ci lines would induce ectopic *wg* RNA in this assay.

Ci5m lines that expressed *ci* RNA at levels at least as high as endogenous *ci* RNA under *prd*GAL4 control induced anterior expansions of *wg* RNA in each of the five cases tested (Figs 5J, 7E; data not shown). Some of these lines expressed no more *ci* RNA than correspondingly tagged wild-type lines that supported only a single-cell-wide *wg* stripe (for example, compare Fig. 5I with 5E). Hence it is clear that Ci5m lines have greater *wg*-inducing activity than corresponding wild-type lines for a given amount of *ci* mRNA.

By searching among the 12% of Ci5m lines that were not embryonic lethal in crosses to *ptc*GAL4, we were able to identify three lines that expressed levels of *ci* RNA lower than or equal to endogenous *ci* (Fig. 5G) and supported mostly single-cell stripes of *wg* expression with no, or very few, instances of anterior expansion (Figs 5H, 8E; data not shown). Thus, Ci5m only induced *wg* transcription in cells adjacent to a source of Hh, indicating that Hh can stimulate Ci5m activity.

Inhibition of PKA increases full-length wild-type Ci concentration, but results in little change in Ci5m

If Ci5m is not proteolyzed to a 75 kDa form at all and if PKA inhibition in wild-type embryos serves only to inhibit this proteolysis, then expression of the PKA inhibitor R* should have no effect on either full-length levels or activity of Ci5m. We examined full-length Ci protein levels encoded by wild-type and mutant *ci* transgenes in embryos lacking endogenous Ci using the 2A1 monoclonal antibody. The level of full-length wild-type protein increased significantly in the presence of R* (Fig. 6A,B and C,D), as previously observed for endogenous

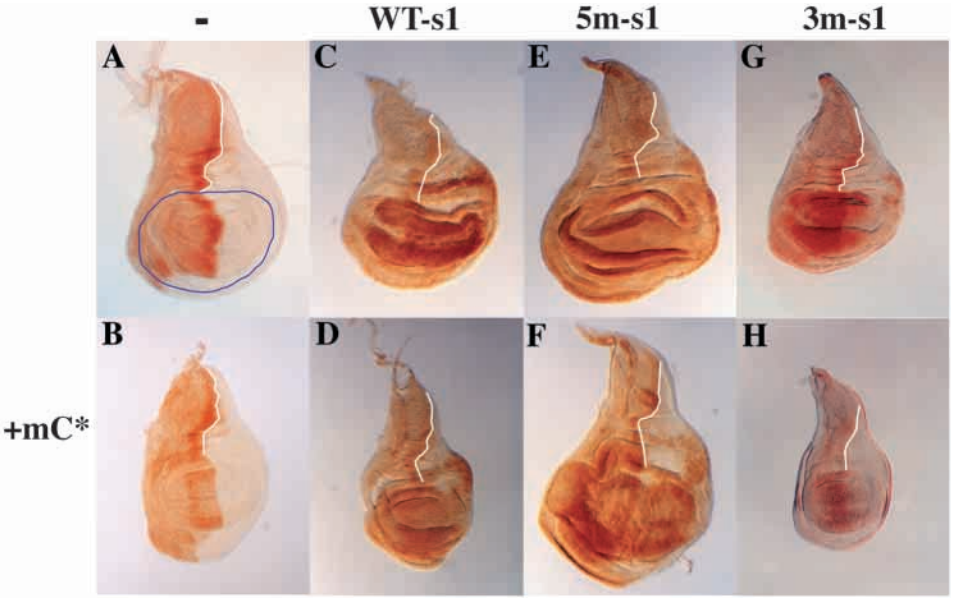

Fig. 4. Ci-155 levels for wild-type Ci and Ci3m, but not Ci5m, can be reduced by increasing PKA activity. Wing imaginal discs from late third instar larvae expressing only endogenous Ci (A,B), or additionally expressing wild-type Ci (C,D), Ci5m (E,F), or Ci3m (G,H) transgenes (from the specific lines indicated) in the wing pouch region were stained with 2A1 monoclonal antibody, which recognizes an epitope C-terminal to the cleavage site for generating Ci-75 (Motzny and Holmgren, 1995). (B,D,F,H) The constitutively active PKA catalytic subunit mC* was co-expressed in the wing pouch region using the *71B* GAL4 line. A blue line in A outlines the area of the wing disc where the transgenes are expressed under *71B* control (Johnson et al., 1995). Discs are oriented with anterior to the left and dorsal up. We have added a white line immediately posterior to high level Ci-155 staining in the region of the disc dorsal to the wing pouch in order to highlight the A/P border.

Ci protein (Ohlmeyer and Kalderon, 1997). In contrast, there was clearly very little change in the level of full-length Ci5m protein in the presence of R* (Fig. 6E,F and G,H). In some lines, R* appeared to induce a small increase in Ci5m levels; however, variability amongst embryos in a given sample precluded a definitive judgement on this issue.

Inhibition of PKA leads to expansions in *wg* expression elicited by wild-type Ci, but not Ci5m

We next looked at the effect of PKA inhibition on *wg* transcription in embryos lacking endogenous *ci*, by expressing transgenic *ci*, with or without the PKA inhibitor R*, in alternating segments. Most wild-type lines supported a single-cell-wide stripe of *wg* RNA in the absence of R* and gave clear anterior expansions of *wg* RNA in the presence of R* in stage 11 embryos (Fig. 7A,B; data not shown). Most Ci5m lines induced expanded *wg* stripes that were not enhanced further by co-expression of R* (Fig. 7E,F; data not shown). PKA inhibition also had no effect on the single-cell-wide stripes of *wg* in embryos from the two weak Ci5m lines that we tested (Figs 7C,D, 8E,F).

We were concerned that the failure of weakly expressing Ci5m lines to respond to R* might be due to insufficient levels of transgenic *ci* because R* also failed to induce ectopic *wg* RNA in a wild-type Ci line (WT-w1) that expressed *ci* at or below endogenous levels (Fig. 8A,B). Previous studies have shown that Su(fu) is a dosage-dependent inhibitor of Ci and that the response of *wg* expression to PKA inhibition in embryos is dramatically enhanced in the absence of Su(fu) (Ohlmeyer and Kalderon, 1998). We therefore tested the effects of R* on the activity of the weakest wild-type and Ci5m lines

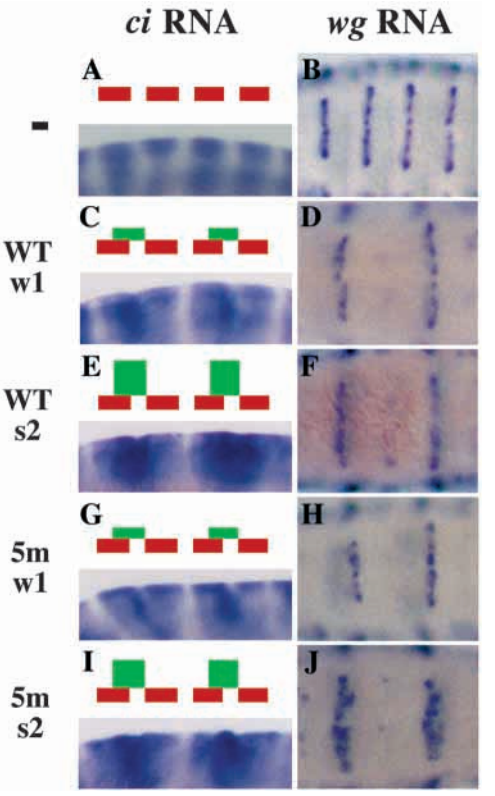

Fig. 5. Ci5m is more active than wild-type Ci transgenes at inducing *wg* transcription. Results of in situ hybridization to *ci* RNA (A,C,E,G,I) and *wg* RNA (B,D,F,H,J) in stage 11 embryos expressing Ci transgenes from the wild-type lines WT-w1 (C,D) and WT-s2 (E,F), or the Ci5m lines 5m-w1 (G,H) and 5m-s2 (I,J) under *prd*GAL4 control. The designation 'w' signifies weak expression, whereas 's' signifies stronger expression, producing *ci* RNA levels in excess of endogenous *ci* RNA in this assay. (A,B) Embryos express only endogenous *ci*; (C,E,G,I) embryos expressing both endogenous and transgenic *ci*; (D,F,H,J) embryos expressing only the transgene. The red and green bars approximate the relative levels and position of endogenous *ci* RNA and *ci* RNA from the transgene, respectively. The *ci* RNA panels show a lateral view and *wg* RNA panels show a ventral view of the posterior two *wg* stripes of stage 11 embryos. Anterior is to the left in all panels.

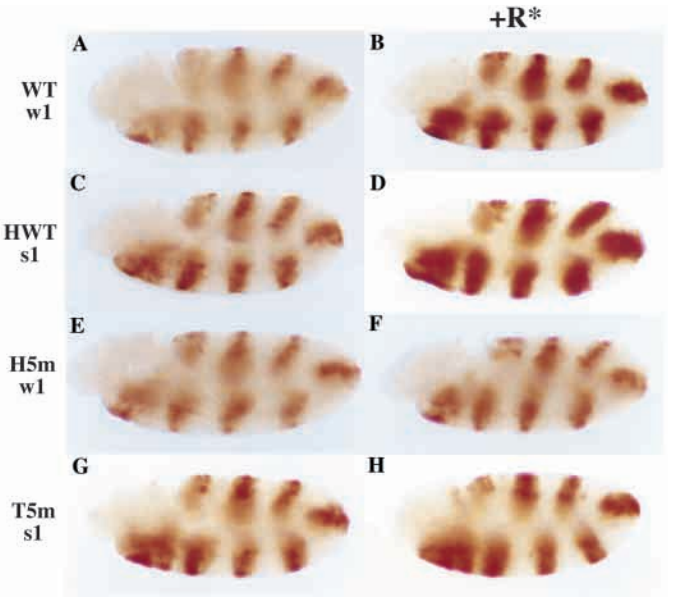

Fig. 6. Inhibition of PKA leads to increased full-length wild-type Ci levels, but has little effect on Ci5m. 2A1 antibody staining of full-length Ci is shown for stage 11 embryos that lack endogenous Ci but express either wild type Ci at low (A,B) or high (C,D) levels, or Ci5m at low (E,F) or high (G,H) levels by using *prd*GAL4 and the indicated transgenic lines, in the absence (A,C,E,G) or presence (B,D,F,H) of the PKA inhibitor R*. Embryos are oriented with anterior to the left.

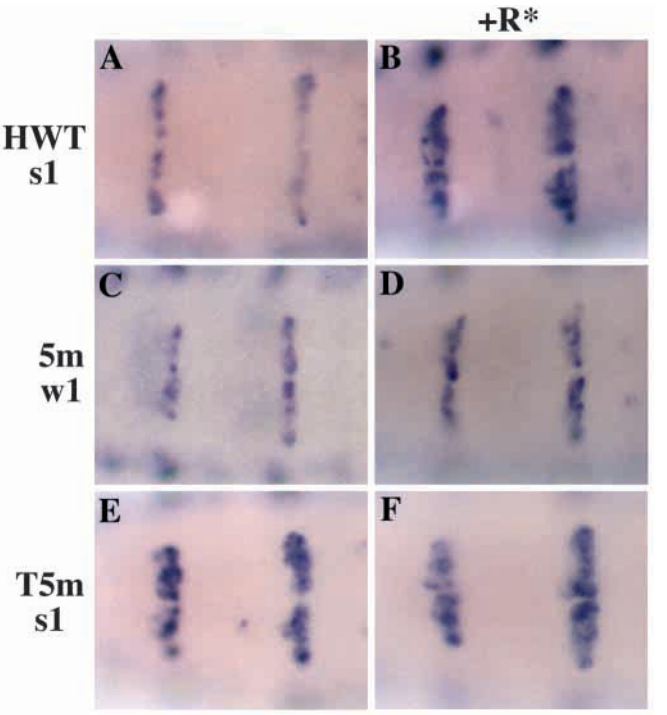

Fig. 7. Inhibition of PKA leads to increased *wg* transcription by most wild-type Ci transgenic lines, but not Ci5m lines. In situ hybridization to *wg* RNA is shown for stage 11 embryos lacking endogenous *ci* but expressing wild-type Ci from the transgenic line HWT-s1 (A,B), or Ci5m from the weak line 5m-w1 (C,D) or from the strong line T5m-s1 (E,F) under *prd*GAL4 control, in the absence (A,C,E) or presence (B,D,F) of the PKA inhibitor R*. A ventral view of the posterior two stripes of wg expression is shown. Embryos are oriented with anterior to the left.

in the absence of maternal Su(fu). Removal of maternal Su(fu) did not change the *wg* expression pattern supported by wild-type transgenic *ci* in the absence of R*, but caused a large expansion of *wg* expression in its presence (Fig. 8C,D). In contrast, the single-cell-wide stripes of *wg* in embryos expressing Ci5m were slightly expanded by the loss of maternal Su(fu) (Fig. 8E,G), but were not further expanded by expression of R* (Fig. 8H). Thus, for every line we tested, the PKA inhibitor R* could cause an expansion in *wg* transcription in embryos expressing wild-type Ci but in no case did R* alter the *wg* expression pattern in embryos expressing Ci5m.

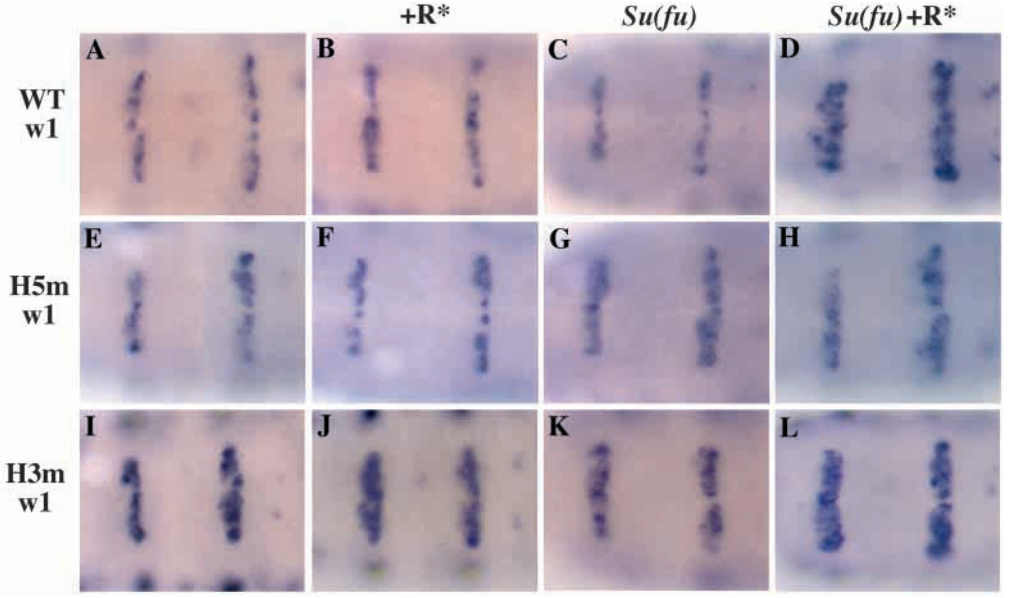

Fig. 8. Loss of maternal Su(fu) reveals induction of ectopic *wg* RNA by PKA inhibition for lines expressing low levels of wild-type Ci or Ci3m, but not Ci5m. *wg* RNA is shown for stage 11 embryos that lack endogenous *ci* but express low levels of wild-type Ci (A-D), Ci5m (E-H), or Ci3m (I-L) using the indicated transgenic lines and *prd*GAL4 in the absence (A,C,E,G,I,K) or presence (B,D,F,H,J,L) of the PKA inhibitor R* in otherwise wild-type embryos (A,B,E,F,I,J) or in embryos lacking maternal Su(fu) (C,D,G,H,K,L). A ventral view of the posterior two stripes is shown, and the anterior of each embryo is oriented to the left. H3m-w1 is a third chromosome line, and so in this cross only half of the embryos expressing transgenic Ci also express R*. We therefore considered embryos with the most-expanded *wg* RNA staining as most likely to be expressing both transgenes.

We examined *wg* expression in two Ci3m lines. In one case, *wg* expression was expanded to a similar degree in the absence and in the presence of R* (data not shown). For the weaker line, *wg* expression was confined to a single-cell width in about half of the stripes and was slightly expanded in the rest (Fig. 8I). In embryos co-expressing Ci3m and R*, the stripes of *wg* expression appeared quite similar to those without R* (Fig. 8J). However, when embryos lacked maternal Su(fu), expansions of *wg* transcription were clearly greater when both R* and Ci3m were expressed than when Ci3m was expressed alone (Fig. 8K,L). Thus, the activity of Ci3m could be increased by PKA inhibition when the transcriptional response was sensitized by eliminating maternal Su(fu).

DISCUSSION

We have shown that the PKA phosphorylation sites in Ci are required for proteolysis of Ci-155 to Ci-75. These sites are also required for PKA inhibition to increase Ci-155 levels and Ci transcriptional activity. These data strongly suggest that PKA directly phosphorylates Ci-155 in vivo to promote proteolysis to Ci-75. It is, however, formally possible that a PKA-responsive protein kinase recognizes the PKA consensus sites or that alteration of these sites affects Ci properties other than its phosphorylation.

Alteration of either three or five consensus PKA phosphorylation sites mimics the effects of reducing PKA activity by eliminating the 75 kDa cleavage product as measured by an immunoprecipitation/western blot assay of stage 10-13 embryos. Unfortunately, the sensitivity of this assay is not great since only a small portion of wild-type Ci protein is in the 75 kDa form. On the basis of this assay alone, we cannot, therefore, rule out the possibility that a very small amount of Ci3m or Ci5m is proteolysed.

Further studies showed that the activity of the five-site mutant, Ci5m, was not altered in response to PKA inhibition and that the level of full-length Ci5m did not respond significantly to PKA hyperactivity or PKA inhibition. These results are consistent with the idea that PKA promotes proteolysis of Ci-155 to Ci-75 solely by acting on the five potential PKA sites of Ci that have been altered in Ci5m.

The three-site mutant, Ci3m, like Ci5m, was clearly more active than wild-type Ci but was distinguishable from Ci5m in several respects. First, the ability of Ci3m lines to induce embryonic or pupal lethality was lower than that of Ci5m lines. Second, for one Ci3m line, *wg* transcription in embryos could be enhanced by PKA inhibition, albeit in a sensitized background. Third, PKA hyperactivity reduced the levels of full-length Ci3m in posterior compartment cells of the wing disc and reduced the Ci3m-induced overgrowth of the disc. These observations did not directly show that Ci3m is proteolyzed to Ci-75, but are most easily explained by proposing that at least one of the two sites altered in Ci5m but not in Ci3m (**A** and **B** in Fig. 1) can be phosphorylated by PKA and promote proteolysis of Ci-155 to Ci-75.

Our studies in animals are essentially in agreement with previous studies in cultured *Drosophila* cells, which showed that a Ci variant lacking the sites we denote **1**, **2**, **3** and **B** was not proteolyzed to a 75 kDa form and was more active than wild-type Ci in promoting transcription from Ci-responsive promoters (Chen et al., 1998). In those assays, the transcriptional activities of some Ci variants lacking only single PKA sites or pairs of sites were very similar to that of the four-site variant. From the collective data, we propose that under physiological conditions, each of the PKA sites **1**, **2**, and **3**, contributes significantly to proteolysis of Ci-155, and that PKA sites **A** and/or **B** promote proteolysis less efficiently.

How does PKA phosphorylation of Ci-155 lead to its proteolysis? Loss of *cos*2 activity in wing disc clones induces high levels of Ci-155 (Sisson et al., 1997), suggesting that the integrity of the multiprotein cytoplasmic complex that contains Ci or the association of this complex with microtubules may be necessary in order for proteolysis to occur. It is possible that Ci phosphorylation also affects proteolysis by altering these interactions. A more direct role for PKA phosphorylation of Ci has been proposed based on the sequence and properties of the Slimb protein, which affects the conversion of Ci-155 to Ci-75 (Jiang and Struhl, 1998). Slimb belongs to a family of F-box/WD40-repeat proteins implicated in binding to and targeting phosphorylated molecules for ubiquitin-mediated degradation (Maniatis, 1999, and references therein). It was recently shown that the vertebrate Slimb homolog, β-TRCP, targets IκB and β-catenin for ubiquitin-mediated degradation by binding specifically to a phosphorylated motif (DSGXXS, where both serines must be phosphorylated) present in both proteins. Whether Slimb participates in such a direct manner in Ci proteolysis is not clear (Theodosiou et al., 1998). Slimb has not been shown to bind to Ci, and Ci proteolysis has not been shown to involve ubiquitination; Ci proteolysis is also unusual in being incomplete, leaving a stable 75 kDa product.

Sequences around PKA sites **1**, **2** and **3** show some extended similarity to each other (consensus RR(D/S)SQXSXXSS; Fig. 1) but are quite different from the IκB and β-catenin consensus. It will be interesting to determine if Slimb, or another F-box protein, can bind directly to these regions of Ci when phosphorylated by PKA. Since Slimb recognition requires phosphorylation at multiple residues and the PKA site consensus in Ci contains additional serines, it is worth considering that the activity of a protein kinase in addition to PKA may also contribute to the regulation of Ci proteolysis.

The antagonistic effect of PKA on Hh signaling is conserved in vertebrates (Fan et al., 1995; Hammerschmidt et al., 1996). It was recently shown that each of the vertebrate GLI proteins can form a product comparable to Ci-75 and that the amount of a C-terminally truncated GLI3 product can be elevated by increasing PKA activity (Ruiz i Altaba, 1999). Given the extensive sequence similarity between Ci and GLI proteins around the sites we have studied, it seems likely that a similar protein domain would recognize the conserved PKA sites in these proteins.

The functional properties of Ci5m and Ci3m together with in vitro assays of Ci phosphorylation by PKA suggest that Ci-155 is normally phosphorylated on at least some of the PKA sites that we have studied in vivo. It will be interesting to determine if Hh signaling affects the phosphorylation status of these sites. Previous studies have shown that Hh can signal normally in cells where PKA activity cannot be regulated transcriptionally or by cAMP, suggesting that Hh signaling does not alter PKA activity (Ingham, 1998). There is some evidence that signaling by Hh family members leads to increased serine/threonine phosphatase activity (Krishnan et al., 1997), potentially leading to dephosphorylation of Ci. However, it is also possible that Hh signaling blocks

conversion of Ci-155 to Ci-75 at a later step without directly affecting Ci phosphorylation.

It has been suggested that the Hh pathway acts on Ci protein in two ways: first, it inhibits proteolysis of Ci, then it converts full-length Ci into a more potent transcriptional activator (Ohlmeyer and Kalderon, 1998; Méthot and Basler, 1999). Our data support this model. First, we showed that high levels of full-length Ci protein are required to induce Hh-independent transcription of target genes. These levels could be achieved by expressing amounts of Ci5m RNA only slightly in excess of physiological levels, presumably because all the resulting protein remains full-length. By contrast, similar amounts of transgenic wild-type *ci* RNA did not induce ectopic *wg* expression unless proteolysis of Ci-155 was blocked by PKA inhibition. Second, low-level expression of Ci5m induced *wg* transcription only in cells adjacent to Hh-expressing cells, demonstrating that the activity of an uncleavable form of Ci can be regulated by Hh. Previous studies have shown that another cleavage-resistant Ci variant, containing a large deletion around the cleavage site, is still Hh responsive in wing imaginal discs (Méthot and Basler, 1999). It has been proposed that Hh stimulates the activity of Ci-155 by opposing the stoichiometric inhibitory influence of Su(fu) (Ohlmeyer and Kalderon, 1998). Consistent with this idea, *wg* stripes induced by low levels of Ci5m extended beyond the range of normal Hh signaling when maternally supplied Su(fu) was eliminated. However, these *wg* stripes were not maximally expanded, leaving open the possibility of additional regulatory input from Hh that is independent of Su(fu) and conversion of Ci-155 to Ci-75.

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