Development 126, 3607-3616 (1999)
Printed in Great Britain © The Company of Biologists Limited 1999
DEV8553

Mutants of *cubitus interruptus* that are independent of PKA regulation are independent of *hedgehog* signaling

Yang Chen[1], Jean-René Cardinaux[1], Richard H. Goodman[1] and Sarah M. Smolik[2,*]

[1]Vollum Institute and [2]Department of Cell and Developmental Biology L-215, Oregon Health Sciences University, 3181 SW Sam Jackson Park Road, Portland, OR 97201, USA
*Author for correspondence (e-mail: smoliks@ohsu.edu)

Accepted 31 May; published on WWW 19 July 1999

SUMMARY

Hedgehog (HH) is an important morphogen involved in pattern formation during *Drosophila* embryogenesis and disc development. *cubitus interruptus* (*ci*) encodes a transcription factor responsible for transducing the *hh* signal in the nucleus and activating *hh* target gene expression. Previous studies have shown that CI exists in two forms: a 75 kDa proteolytic repressor form and a 155 kDa activator form. The ratio of these forms, which is regulated positively by *hh* signaling and negatively by PKA activity, determines the on/off status of *hh* target gene expression. In this paper, we demonstrate that the exogenous expression of CI that is mutant for four consensus PKA sites [CI(m1-4)], causes ectopic expression of *wingless* (*wg*) in vivo and a phenotype consistent with *wg* overexpression. Expression of CI(m1-4), but not CI(wt), can rescue the *hh* mutant phenotype and restore *wg* expression in *hh* mutant embryos. When PKA activity is suppressed by expressing a dominant negative PKA mutant, the exogenous expression of CI(wt) results in overexpression of *wg* and lethality in embryogenesis, defects that are similar to those caused by the exogenous expression of CI(m1-4). In addition, we demonstrate that, in cell culture, the mutation of any one of the three serine-containing PKA sites abolishes the proteolytic processing of CI. We also show that PKA directly phosphorylates the four consensus phosphorylation sites in vitro. Taken together, our results suggest that positive *hh* and negative PKA regulation of *wg* gene expression converge on the regulation of CI phosphorylation.

Key words: *Drosophila*, *hedgehog*, *wingless*, *cubitus interruptus*

INTRODUCTION

hedgehog (*hh*) and *wingless* (*wg*) are two segment polarity genes whose initial expression is triggered by pair-rule gene products and maintained by segment polarity gene products. The initial *wg* and *hh* expression is activated by the concerted function of zygotic gene products in late cellular blastoderm (DiNardo et al., 1988; Lee et al., 1992; Tabata et al., 1992). Segment polarity gene products, including *wg* and *hh* themselves, maintain *wg* and *hh* gene expression and a mutually dependent regulatory relationship between *wg* and *hh* develops during germband extension (Martinez Arias et al., 1988; DiNardo et al., 1988).

The *hh* signal transduction cascade is one of the best studied signaling pathways involved in embryogenesis and disc development. The current model proposes that HH is secreted from the posterior compartment of each segment, binds its postulated receptor, Patched (PTC) (Johnson et al., 1995; Marigo et al., 1996), and relieves the PTC inhibition of Smoothened (SMO) activity (Alcedo et al., 1996; Heuvel and Ingham, 1996). The *smo* pathway then activates the transcription factor CI resulting in the activation of *hh* target gene expression. Genetic studies have shown that PKA and *costal-2* (*cos*-2) negatively regulate this pathway (Alcedo et al., 1996; Heuvel and Ingham, 1996; Jiang and Struhl, 1995; Lepage et al., 1995; Li et al., 1995; Ohlmeyer and Kalderon, 1997; Pan and Rubin, 1995) and that *fu* has a positive effect (Forbes et al., 1993). Subsequent studies suggest that *hh* signaling stimulates the *fu* kinase activity that causes the maturation of full-length CI into a transient transcriptional activator (Ohlmeyer and Kalderon, 1998). This work also suggests that the role of *Su(fu)* is to antagonize this process. *wg* is one of the *hh* target genes. Upon secretion from the anterior compartment, WG acts posteriorly to maintain *hh* expression; thus *hh* and *wg* form a positive feedback loop around the anterior-posterior (A/P) boundary of the segment, reinforcing each other's expression and orchestrating the patterning events after gastrulation (DiNardo et al., 1988).

Genetic and biochemical experiments have shown that CI is the transcription factor responsible for transducing the *hh* signal into the nucleus (Alexandre et al., 1996; Dominguez et al., 1996; Ohlen et al., 1997). A perplexing phenomenon associated with CI as a transcription factor is that the activated, presumably full-length, form has never been detected in the nucleus in vivo, not even in the nuclei of cells located at the A/P boundary, where CI exerts its transactivation function, or in mutants (e.g.: *ptc*, *cos2*, PKA mutants) that exhibit elevated levels of CI protein and CI target-gene expression (Motzny and

Holmgren, 1995; Slusarski et al., 1995). One accepted explanation for this phenomenon is that cells require undetectable or transient amounts of activated CI in the nucleus to activate target gene expression. CI exists as two forms; a 155 kDa transcriptionally active form, and a 75 kDa N-terminal proteolytic fragment, which contains the zinc finger DNA-binding domain and is a repressor of *hh* target gene expression (Aza-Blanc et al., 1997). Both forms are detected in a large cytosolic complex that includes COS-2, FU and additional unidentified proteins (Aza-Blanc et al., 1997; Robbins et al., 1997; Sisson et al., 1997). In the absence of a *hh* signal, CI is proteolysed and the N-terminal fragment of CI can be detected in the nucleus. When cells receive a HH signal, the CI complex disassociates from the microtubules, CI is not proteolysed and the activator, presumably full-length, form of CI may then activate *hh* target gene expression.

Recently, we have demonstrated in Kc cells that PKA exerts its negative effect on CI activity by promoting the proteolysis of the CI protein (Chen et al., 1998). In this paper, we demonstrate that a CI protein that is mutant for consensus PKA phosphorylation sites can bypass the *hh* regulation of *wg* gene expression. Furthermore, we show that the mutation of any one of the three serine-containing consensus PKA sites suppresses the proteolytic processing of CI and that PKA directly phosphorylates these sites in vitro. Our data suggest that the *hh* signaling cascade and PKA regulation of gene expression converge on the CI phosphorylation status in vivo.

MATERIALS AND METHODS

Plasmids

pPac-PKA, pPac-PKI, pPac-HACI(wt), pPac-HACI(m1-4), pPac-luciferase, ADHCAT/GLI6BS and pPac-CI PKA mutants have been described previously (Chen et al., 1998). pPac-HACI(m1), pPac-HACI(m2), pPac-HACI(m3) and pPac-HACI(m4) were made by inserting hemagglutanin (HA) tag into the *Mlu*I site at the 5th amino acid in corresponding CI PKA mutant that had Ser-838, Ser-856, Ser 892 and Thr-1006 mutated to alanine, respectively, using site-directed mutagenesis kits from Promega**.** pUAST-HACI(wt) and pUAST-HACI(m1-4) were made by inserting the *Bam*HI-*Not*I fragment containing HACI(wt) or HACI(m1-4) into the pUAST vector (Brand and Perrimon, 1993) that had been digested with *Bgl*II and *Not*I. The GST-CI 441-1065 (wt) and GST-CI 441-1056 (m1-4) constructs used to generate the tryptic phosphopeptide maps were made by inserting a filled, *Eco*RV-*Spe*I fragment of either CI(wt) or CI(m1-4) into the pGEX-KG vector (Pharmacia) that had been digested with *Eco*RI and filled. The GST/CI junctions were sequenced to determine that the coding sequences were in frame.

Tissue culture, transfection and immunoprecipitation

Kc cells were maintained, transfected and assayed for luciferase and CAT activities as described (Chen et al., 1998). 15 μg of total DNA (100 ng of pPac-luciferase, 5 μg of ADHCAT/GLI6BS reporter gene, 2 μg of pPac-CI construct, 4 μg of pPac-PKA or PKI, plus 4 μg of pPac, or various amounts of pPac-CI construct as indicated in the figures) were transfected. Cl-8 cells (kindly provided by T. Kornberg, University of California, San Francisco) were maintained as described by van Leeuwen et al. (1994). S2 cells that stably express N-terminal HH protein (N-hh) were provided by J. M. Bishop (University of California, San Francisco) and were maintained as described (Therond et al., 1996). Fly extract was prepared as described by Currie et al. (1988). For transfections, approximately 2×10^5 cells were seeded in 24-well dishes. A total of 3 μg of DNA (20 ng of pPac-luciferase, 1 μg of ADHCAT/GLI6BS reporter gene, different amounts of pPac-CI construct, plus 2 μg of pPac) were transfected using calcium phosphate transfection kits from GIBCO/BRL. Both Kc and Cl-8 cells were allowed to recover for 24 hours in N-hh or control medium before they were assayed for luciferase and CAT activities. Protocols for immunoprecipitations and western blots are described by Chen et al. (1998). Each dose-response curve was repeated at least two times and was done in triplicate.

Phosphorylation of GST-CI constructs and phosphopeptide mapping

Recombinant GST-CI 441-1065 (wt) and GST-CI 441-1065 (m1-4) proteins (approximately 1 μg) were phosphorylated in vitro with the recombinant catalytic subunit of PKA in the presence of [γ-^{32}P]ATP. The reaction mixtures containing 250 μM of [γ-^{32}P]ATP (5000 cpm/pmole), 50 mM MOPS [pH 6.8], 50 mM NaCl, 2mM $MgCl_2$, 1 mM DTT, 1.0 nM PKA, were incubated for 45 minutes at 30°C. Phosphorylated GST-CI 441-1065 proteins were separated by 6% SDS-PAGE, and identified by autoradiography of the dried gel. For two-dimensional phosphopeptide mapping, the phospho-GST-CI bands were cut from the gel and two-dimensional tryptic phosphopeptide maps were performed on the extracted proteins as described (Boyle et al., 1991).

Drosophila stocks and culture conditions

The *prd-GAL4* strain RG1 (Yoffe et al., 1995) and the *UAS-R** strain (Ohlmeyer and Kalderon, 1997) were kindly provided by Dan Kalderon (Columbia University). The *hedgehog* allele hh^{IJ} was obtained from the Umea Stock Center. The hh^{IJ} allele is a null or near amorphic allele of *hh* that has been used to study the activity of exogenously expressed CI in other studies (Ingham and Hidalgo, 1993). We chose this allele in order to directly compare our results with those previously reported. The hh^{IJ} *prd-GAL4* recombinant chromosome was generated using standard genetic techniques. The balancer chromosomes *TM2*, *TM3* and *MKRS* that are used in these studies can be found in Lindsley and Zimm (1992). The wild-type strain used in these studies is Canton-S. Flies were cultured on standard *Drosophila* cornmeal-yeast-source medium in 8 oz plastic bottles or 28×95 mm plastic shell vials. All crosses were reared at 25°C.

Germline transformation and cuticle preparations

The HACI(wt) and HACI(m1-4) pUAST constructs were injected at a concentration of 400 mg/ml into *y w* embryos as described (Spradling, 1986) and using 100 mg/ml pπΔ2-3 (kindly provided by J. Posakony) as a helper plasmid. At least four independent transformants were generated for each HACI construct and tested for viability and expression with the *prd-GAL4* line.

UAS-HACI(m1-4) flies were crossed to *prd-GAL4/MKRS* flies and *UAS-HACI(wt or m1-4);* hh^{IJ}*/TM2* flies were crossed to hh^{IJ} *prd-GAL4/MKRS* flies. The matings were allowed to proceed for 24-48 hours. Likewise, control hh^{IJ}*/TM2* and hh^{IJ} *prd-GAL4/MKRS* flies were allowed to mate for 24-48 hours. The eggs were then collected after a 16-24 hour period and aged for an additional 48 hours. The unhatched embryos were collected, dechorionated in 50% bleach, rinsed in phosphate-buffered saline (PBS) and the vitelline membranes removed in 1:1 heptane: methanol. They were washed in 100% ethanol and mounted as described (Lewis, 1978). The cuticles were visualized by dark-field optics and photographed with Tech Pan 50 film.

For the viable Canton-S embryos and those from the cross between *UAS-HACI(wt)* flies and *prd-GAL4/MKRS* flies, the flies were allowed to lay eggs for 3 hours. The eggs were collected and aged for 24 hours when they were processed as described.

Whole-mount embryo in situ hybridization and immunostaining

The embryos were collected after a 3 hour egg lay period, then subjected

to heat shock at 29°C for 5 hours to induce the expression of GAL4 activator. The immunohistochemistry of whole-mount embryos was carried out as described by Rose et al. (1997) and the in situ hybridization protocols were those of Tautz and Pfeile (1989) and Fleming et al. (1990). The double staining was performed as described above by first performing HA staining followed by in situ hybridization. The anti-HA antibody (Boehringer Mannheim) was first diluted 1:10 in 10% horse serum/PBS and preincubated with Canton-S embryos overnight at 4°C. For HA staining only, a final concentration of 1:500 was used. For in situ coupled immunostaining, a final concentration of 1:200 was incubated with embryos at room temperature for 1 hour.

The *wg* mRNA probe was kindly provided by M. Forte (Vollum Institute) and was used at 1:5000 dilution. The embryos were visualized by Nomarski optics and photographed with either Ektachrome 64 or Tech Pan 50 film.

RESULTS

Phosphorylation of all three PKA sites in CI is required for its proteolysis

We have shown previously that PKA regulates CI activity and promotes its proteolysis in tissue culture, thus providing a mechanism for the negative regulation of the *hh* signal transduction pathway by PKA (Chen et al., 1998). To investigate the relative importance of the four potential PKA phosphorylation sites in CI-mediated transactivation and CI processing, we mutated the serine of each site singly to an alanine and transfected the mutant constructs into Kc cells to examine the transcriptional activity and proteolytic processing of the mutant proteins. As shown in Fig. 1A, mutating any one of the first three consensus PKA phosphorylation sites increases CI transcriptional activity. The fold increase over wild-type CI activity from m1, m2 and m3 is comparable to that of the CI(m1-4) protein that is mutant for all four sites. The fourth PKA site is not important because m4 activity is equivalent to wild-type CI activity and the m4 CI protein is proteolysed to the same degree as the wild-type CI protein (Fig. 1B). When the PKA catalytic subunit is co-transfected with the CI PKA mutants, m1, m2 and m3, the same positive two-fold effect is observed as that seen for the CI(m1-4) protein (Chen et al., 1998). Like the wild-type CI, the transcriptional activity of the CI m4 mutant decreases in the presence of exogenous

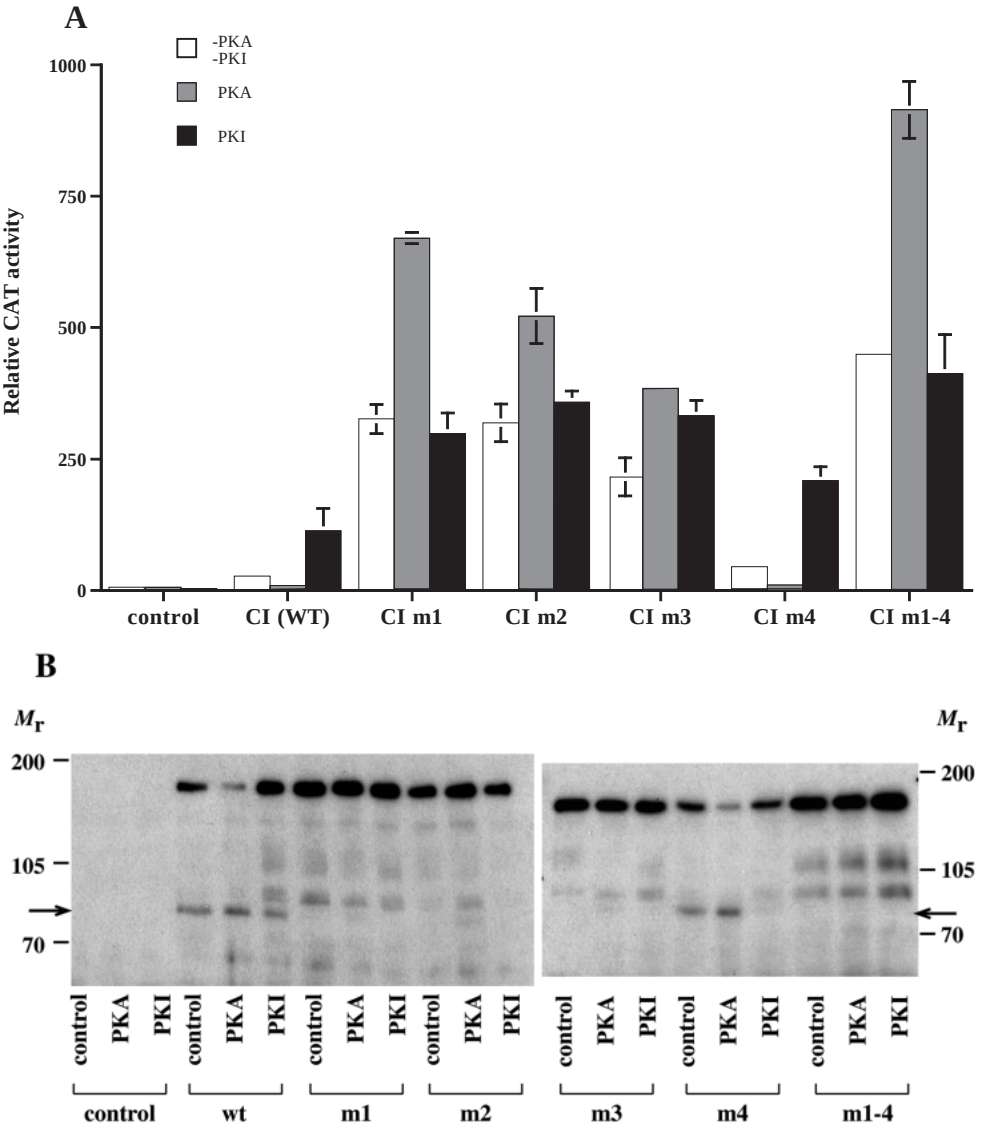

Fig. 1. Effect of individual PKA sites on CI-mediated transcriptional activation and proteolysis. (A) 100 ng of pPac-luciferase, 5 μg of the ADHCAT/GLI6BS reporter gene, 4 μg of pPac-PKA, pPac-PKI or pPac and 2 μg of the indicated pPac-HACI or pPac (control) construct were transfected into Kc cells. CAT activities were normalized to the corresponding luciferase activities. Data represent means ± s.e.m. (B) A mixture of 10 μg of the indicated pPac-HACI constructs and 10 μg of pPac, or pPac-PKA or pPac-PKI were transfected into Kc cells. HA-tagged proteins were immunoprecipitated with a rat anti-HA monoclonal antibody. Western blots of the immunoprecipitates were then probed with the anti-HA monoclonal antibody. Arrows indicate the position of the 75 kDa repressor form of CI. The levels of the intermediate bands are variable from experiment to experiment (compare to Fig. 2C and 2D inserts and Chen et al., 1998) and we presume that they represent breakdown products.

PKA. When PKI, a PKA inhibitor, is co-transfected with the different CI mutant constructs, we do not observe any further increase in CI activity, indicating that a mutation of any one of the first three PKA phosphorylation sites has the same effect as mutating all the sites. However, inhibition of PKA activity by PKI increases m4 activity, which is expected if the fourth site does not contribute significantly to the regulation of CI activity.

We then determined the effect of mutating single PKA sites on the proteolytic processing of the CI protein. We expected that the enhanced transcriptional activity of the mutant CI constructs was due to the inability of these proteins to be proteolysed to the 75 kDa repressor. As shown in Fig. 1B, mutating of any one of the first three PKA sites abolishes the proteolysis of CI, suggesting that the phosphorylation of all three PKA sites is required for the processing of CI. Consistent with our transfection data, mutation of the last PKA site does not affect CI proteolysis and m4 processing is regulated in the same way as wild-type CI, i.e. PKA promotes and PKI inhibits m4 proteolysis.

Mutations in the PKA phosphorylation sites of CI can bypass the requirement for *hh* signaling to activate reporter gene expression in cell culture

We have shown that PKA negatively regulates the activity of CI by promoting its proteolysis to the 75 kDa repressor form. We wanted to determine whether *hh* signaling along the A/P boundary of the segments regulates the activity of CI by regulating its phosphorylation state. Reports by Ohlmeyer and Kalderon (1997) suggest that *hh* signaling has a positive effect on *wg* expression that is independent of CI protein modifications. In this case, we would expect that the activity of the CI(m1-4) mutant protein would be augmented in the presence of *hh* signaling. If the primary function of *hh* signaling were to modulate CI phosphorylation, then the activity of the CI(m1-4) mutant protein would be independent of a HH signal. Experiments were performed in Cl-8 cells, which are derived from *Drosophila* disc cells and can respond to a HH signal (Aza-Blanc et al., 1997; Therond et al., 1996).

We transfected Cl-8 cells with CI(m1-4) and CI(wt) constructs and treated them with N-hh-conditioned media to see whether HH could affect the activity of CI that is mutant for the putative PKA phosphorylation sites. As shown in Fig. 2A, transfection of CI(wt) increases the expression of a CI-responsive reporter gene in a dose-dependent manner when grown in the presence of control S2 cell-conditioned medium. Growth in N-hh-conditioned S2 medium causes a further increase in CI transcriptional activity, indicating that CI(wt) activity is increased by HH activity in Cl-8 cells. When the CI(m1-4) construct is introduced into Cl-8 cells, the dose-dependent increase in CI activity is larger than that of CI(wt). Furthermore, when cells transfected with CI(m1-4) are treated with N-hh-conditioned medium, we do not observe any additional increase in transcriptional activity, suggesting that the HH-mediated increase in CI activity may be, at least in part, through dephosphorylation of CI. Endogenous CI does not contribute to these activities as the reporter is not expressed significantly either in the presence or absence of HH (0 points in Fig. 2A,B)

Because the Kc cell line displays PKA-dependent proteolytic mechanisms, we determined whether the *hh* signaling cascade was intact in this cell line as well. As shown in Fig. 2C, N-hh-conditioned medium increases the activity of CI(wt) in Kc cells. Furthermore, the N-hh-conditioned medium inhibits the proteolysis of CI(wt) (Fig. 2C insert) suggesting that the Kc cell line can respond to the HH signal and recapitulate the HH regulation of CI transcriptional activity. When Kc cells are transfected with increasing amounts of CI(m1-4), we observe an increase in CI transcriptional activity

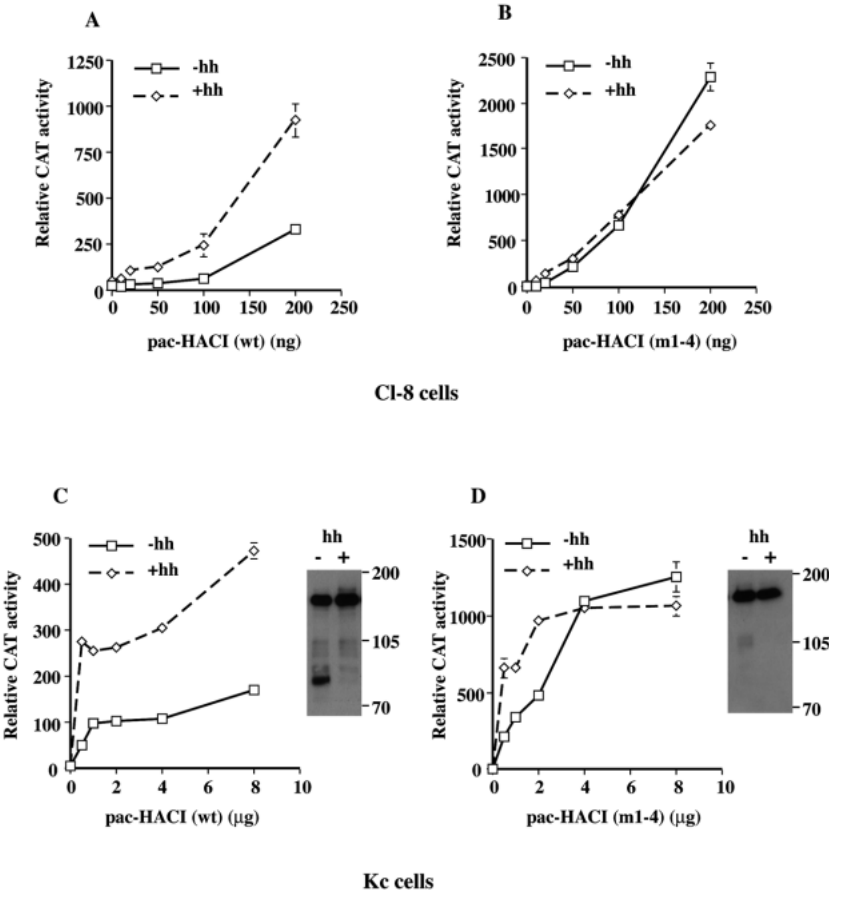

Fig. 2. Effect of *hh* and PKA on CI-mediated transcriptional activation in Cl-8 and Kc cells. (A) Cl-8 cells were transfected with 20 ng of pPac-luciferase, 1 μg of the ADHCAT/GLI6BS reporter gene and increasing amounts of pPac-HACI(wt) (from 0-200 ng) and grown in the presence or absence of N-hh-conditioned medium. (B) Cl-8 cells were transfected as described except that increasing amounts of pPac-HACI(m1-4) (from 0-200 ng) were used. (C) Kc cells were transfected with 100 ng of pPac-luciferase, 5 μg of the ADHCAT/GLI6BS reporter gene and increasing amounts of pPac-HACI(wt) (from 0-8 μg). Cells were treated as described for A. (D) Kc cells were transfected as described for C, except that increasing amounts of pPac-HACI(m1-4) (from 0-8 μg) were used. Inserts for C and D represent western blots of immunoprecipitations from Kc cells transfected with 10 μg of pPac-HACI(wt) (insert C) or pPac-HACI(m1-4) (insert D) treated with or without N-hh-conditioned medium.

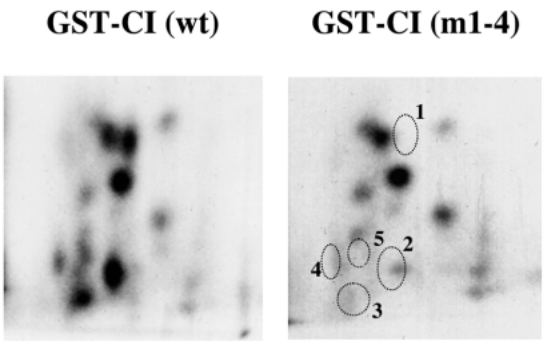

Fig. 3. Two-dimensional phosphopeptide mapping of GST-CI 441-1065 (wt) and (m1-4) proteins phosphorylated by PKA. Recombinant GST-CI 441-1065 (wt) and GST-CI 441-1065 (m1-4) were phosphorylated in vitro by PKA in the presence of [γ-^{32}P]ATP. The phosphopeptides were generated as described and visualized by autoradiography. Five spots are missing in the GST-CI 441-1065 (m1-4) phosphopeptide map (dotted circles).

that is independent of HH. Furthermore, this mutant protein is not proteolysed in the presence of N-hh-conditioned medium (Fig. 2D insert). Thus the regulation of exogenously expressed CI by the *hh* signaling pathway is qualitatively the same in both the Kc and Cl-8 cell lines.

PKA can directly phosphorylate the CI consensus PKA sites

While our experiments demonstrate that PKA cannot regulate a CI protein that is mutant for three of the consensus PKA sites (Chen et al., 1998), we did not determine that PKA directly phosphorylated these sites. To test that PKA directly targets these sites in vivo, it is necessary to follow the phosphorylation of CI throughout development and in mutant backgrounds with antibodies directed against the phosphorylated form of CI. In the absence of these reagents, however, we can determine whether PKA phosphorylates these sites in vitro. We fused the CI fragments of CI(wt) and CI(m1-4) that contain the four PKA sites (aa441-1065) to GST, and generated two-dimensional tryptic phosphopeptide maps of the expressed fusion proteins. The two maps are presented in Fig. 3. There are at least 13 phosphopeptides that are labeled by PKA in the wild-type CI peptide. In vitro, PKA can recognize RxS/T, the subset RRxS/T, RxxS/T and RKxxS/T. The phosphorylation of S is preferred 40:6 over T (reviewed in Pearson and Kemp, 1991) and in vivo, the RRxS site is preferred 2:1 over the others (reviewed in Kennelly and Krebs, 1991). We originally chose to mutate the four consensus RRxS/T sites in CI because they would probably be the preferred phosphorylation sites in vivo. Scanning the CI fragment for all possible consensus PKA sites, we found that all of the phosphopeptides can be accounted for by the number of PKA consensus sites in the fusion protein. Three of the strong spots and two weaker spots that are present in the wild-type fragment are missing in the mutant fragment, demonstrating that PKA can specifically and directly phosphorylate the four RRxS/T consensus PKA sites in vitro. The two weak spots are difficult to distinguish and may represent only one spot or incomplete digestion of a single peptide. GST alone was not phosphorylated (data not shown).

Expression of CI(m1-4) increases *wg* expression and generates a cuticular phenotype consistent with *wg* overexpression

To confirm our cell culture observations and to study the relationship between the CI proteins mutant for consensus PKA sites and *hh* signaling in vivo, we made transgenic flies that have *UAS-HACI* [hemagglutanin(HA) tagged CI (wt or m1-4)] incorporated into the genome and determined whether these transgenes could affect *wg* expression. Four different *UAS-HACI(wt)* and four different *UAS-CI(m1-4)* fly lines were crossed with *paired-GAL4/MKRS* flies, and the resulting *UAS-HACI(wt or m1-4)/+*; *paired-GAL4/+* embryos ectopically express CI(wt) or CI(m1-4) to the same extent in the *paired* (*prd*) domain as assessed by HA antibody staining. Because all of the *ci* transformants have an HA tag at the N terminus of the protein, we are able to distinguish the exogenous from endogenous CI expression by staining the embryos with an anti-HA antibody. While all of the *UAS-HACI(wt)/+*; *prd-GAL4/+* embryos survive to adulthood, only 10% of the expected *UAS-HACI(m1-4)/+*; *prd-GAL4/+* embryos survive past the first instar. As shown in Fig. 4A-D, exogenous HACI is expressed in the broad, alternating segmental stripes that span the *engrailed* (*en*) and *wg* expression domains and define the *prd* domains. In cell culture, CI(m1-4) has a 10-fold increase in transcriptional activity over that of CI(wt). To determine whether the CI(m1-4) protein can increase *hh* target gene expression in vivo, we examined *wg* mRNA expression in the *UAS-HACI(wt)/+; prd-GAL4/+* and *UAS-HACI(m1-4)/+*; *prd-GAL4/+* embryos. Overexpression of CI(wt) in the alternate *prd* stripes does not cause any significant change in *wg* expression in the majority of the embryos examined (Fig. 4C). 93% (200/216) of the embryos double-stained with an HA antibody and *wg* mRNA do not show any increase in *wg* expression. In contrast, the ectopic expression of HACI(m1-4) in the *prd* stripes results in a dramatic anterior expansion of *wg* expression (Fig. 4D). 97% (133/137) of the embryos double-stained with HA antibody and *wg* mRNA shows an increase in the *wg* expression domains. Although the cells both anterior and posterior to the A/P boundary express HACI(m1-4) protein, *wg* expression only expands anteriorly, consistent with the previous finding that only anterior compartment cells are competent to express *wg* (Ingham, 1993). The expansion in *wg* expression caused by the ectopic activity of CI(m1-4) is very obvious (Fig. 4D) when compared with the neighboring segments that express only endogenous CI.

We expected that the cuticular phenotype of the *UAS-HACI(wt)/+; prd-GAL4/+* embryos would be wild type because the *wg* expression in these animals is wild type. All of the embryos from the mating of *UAS-HACI(wt)* flies and *prd-GAL4/MKRS* flies have a wild-type phenotype, and we could not determine the exact genotype of the embryos (Fig. 4E). Because the *UAS-HACI(m1-4)/+; prd-GAL4/+* embryos are virtually lethal, cuticles of unhatched embryos from the cross of *UAS-CI(m1-4)* flies to *prd-GAL4/MKRS* flies were assessed for their phenotype. The *UAS-HACI(m1-4)/+; prd-GAL4/+* embryos have cuticular defects consistent with the overexpression of *wg*. In alternating segments, the posterior rows of denticles contain patches of naked cuticle and the anteriormost rows of the denticle belts are missing denticles (Fig. 4F).

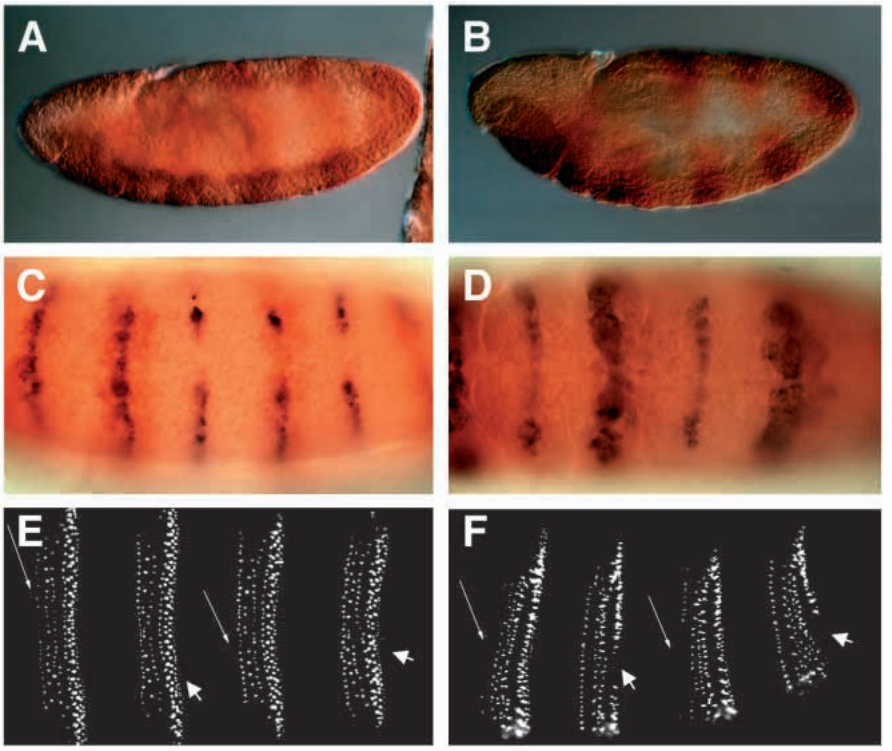

Fig. 4. Expression of HACI(wt) and HACI(m1-4) in the *prd* domain. *UAS-HACI(wt)/+; prd-GAL4/+* (A) and *UAS-HACI(m1-4)/+; prd-GAL4/+* (B) embryos stained with an anti-HA antibody. In both, the HACI protein is expressed to the same extent in the broad stripes characteristic of the *prd*-GAL4 expression pattern. Anterior is to the left and dorsal is up. *UAS-HACI(wt)/+; prd-GAL4/+* (C) and *UAS-HACI(m1-4)/+; prd-GAL4/+* (D) embryos stained with the anti-HA antibody and *wg* RNA probes. The *wg* expression in the HACI(wt) stripes is comparable to the *wg* expression in the alternate domains that express endogenous CI. The *wg* expression in the *prd* stripes expressing HACI(m1-4) is broadened to fill the anterior portion of the *prd* domain. The alternate domains that express only endogenous CI have a wild-type level of *wg* expression that serves as an internal, wild-type control. Anterior is to the left and ventral is forward. The cuticles of *UAS-HACI(wt)/+; prd-GAL4/+ embryos* (E) are wild type. The cuticles of *UAS-HACI(m1-4)/+; prd-GAL4/+* embryos (F) exhibit some defects; alternate segments have missing denticles in the anterior (long arrows) and posterior rows (short arrows). Anterior is left and ventral is forward

CI(m1-4) can rescue the *hh* mutant phenotype

Our cell culture experiments showed that, in Cl-8 cells, the transactivation of the CI reporter plasmid by the CI(m1-4) protein is completely independent of a HH signal. Using the *UAS-HACI* transgenic flies, we asked whether a change in the CI consensus PKA phosphorylation sites is all that is required for HH to signal in vivo. To test this hypothesis, we crossed *UAS-HACI (wt or m1-4); hh^{IJ}/TM2* flies with *prd-GAL4 hh^{IJ}/MKRS* flies and assessed the ability of the CI transgenes to suppress the mutant *hh* phenotype in the *prd* domains of expression.

As a control, *hh^{IJ}/TM2* flies were mated with *prd-GAL4 hh^{IJ}/MKRS* flies and the progeny characterized with respect to *wg* expression and cuticular phenotype. As expected, 25% of the embryos are defective in maintaining *wg* expression during germband extension (Fig. 5C). The remaining 75% of the embryos had a wild-type *wg* expression pattern. Because *prd-GAL4 hh^{IJ}/hh^{IJ}* embryos are lethal, cuticle preparations were made of the unhatched embryos from the control mating. These embryos lack naked cuticle and develop a lawn of randomly arrayed denticles (Fig. 5D). The presence of *prd* on the *hh^{IJ}* chromosome did not affect the *hh^{IJ}* phenotype.

In the population of embryos from the cross between *UAS-HACI(wt); hh^{IJ}/TM2* flies and *hh^{IJ} prd-GAL4/MKRS* flies, approximately 25% of the animals did not express *wg* in germband-extended animals. About 75% had the wild-type *wg* expression pattern and <5% had alternating broad stripes of *wg* expression. This result is consistent with the double-labeling experiments presented above. Thus, exogenous expression of CI(wt) driven by *prd*-GAL4 does not restore *wg* expression in *hh* mutant embryos and the primary phenotype of the *UAS-HACI(wt)/+; hh^{IJ}/hh^{IJ} prd-GAL4* animals is the same as *hh^{IJ}/hh^{IJ} prd-GAL4* animals (Fig. 5E). Because *prd-GAL4 hh^{IJ}/hh^{IJ}* embryos are lethal, cuticle preparations were made of the unhatched embryos from this mating. As shown in Fig. 5F, exogenously expressed CI(wt) does not rescue the *hh* mutant phenotype.

On the contrary, exogenous expression of CI(m1-4) in the *prd* domain restores *wg* expression in that domain (Fig. 5G) and does rescue the *hh* mutant phenotype (Fig. 5H). In the population of embryos from the cross between *UAS-HACI(m1-4); hh^{IJ}/TM2* flies and *hh^{IJ} prd-GAL4/MKRS* flies, 50% of the embryos had a wild-type *wg* expression pattern (*UAS-HACI(m1-4)/+; TM2/MKRS* and *UAS-HACI(m1-4)/+; hh^{IJ}/MKRS* animals), 25% had alternating wild-type and broad bands of *wg* expression in germband-extended embryos (*UAS-HACI(m1-4); hh^{IJ} prd-GAL4/TM2* animals) and 25% had only broad alternating bands of *wg* expression in germband-extended embryos (*UAS-HACI(m1-4); hh^{IJ}/hh^{IJ} prd-GAL4* animals). We never observed embryos without *wg* expression in this population. From the double-labeling experiments, we know that the broad alternating stripes only occur in the presence of the *UAS-HACI* and *prd-GAL4* chromosomes. In the population of embryos from the cross between *UAS-HACI(m1-4); hh^{IJ}/TM2 flies* and *hh^{IJ} prd-GAL4/MKRS* flies, 50% of the embryos live and 50% are lethal either because they are *hh^{IJ} prd-GAL4/hh^{IJ}* or they are heterozygous for the *UAS-HACI(m1-4)* and *hh^{IJ} prd-GAL4* chromosomes. One half of the lethal embryos have the phenotype characteristic of *UAS-HACI(m1-4)/+; hh^{IJ} prd-GAL4/+* transheterozygotes. The other half are *UAS-HACI(m1-4); hh^{IJ}/hh^{IJ} prd-GAL4* embryos and have a suppressed *hh^{IJ}* phenotype; they develop naked cuticle and the bordering denticles exhibit some segmental organization. This phenotype is never observed in the control *hh^{IJ}/hh^{IJ} prd-GAL4* lethal embryos and only rarely (<5%) in the *UAS-HACI(wt); hh^{IJ}/prd-GAL4 hh^{IJ}* lethal embryos. The ability of CI(m1-4) to maintain *wg* expression in the absence of HH signaling suggests that *hh*-dependent target gene expression may be mediated through dephosphorylation of CI.

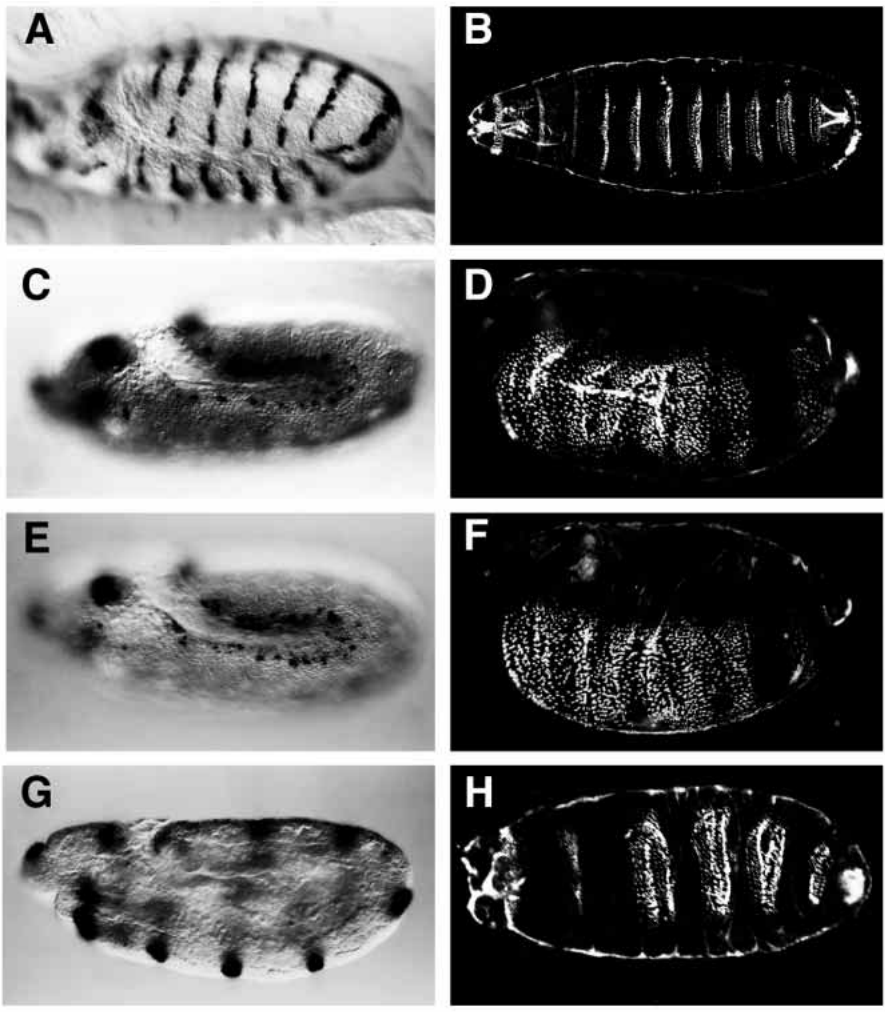

Fig. 5. HACI(m1-4) but not HACI(wt) can rescue the *hh* mutant phenotype in their expression domains. The *wg* expression pattern (A) and cuticle (B) of Canton-S embryos. The *wg* expression pattern (C) and cuticle (D) of *prd-GAL4* hh^{IJ}/hh^{IJ} embryos. As expected, no *wg* expression is detected in the germband-extended *wg* stripes and no naked cuticle is made. The *wg* expression pattern (E) and cuticle (F) of *UAS-HACI(wt)/+; prd-GAL4* hh^{IJ}/hh^{IJ} embryos. HACI(wt) cannot increase *wg* in the homozygous hh^{IJ} embryos nor can it rescue the hh^{IJ} mutant cuticular phenotype. The *wg* expression pattern (G) and cuticle (H) of *UAS-HACI(m1-4)/+; prd-GAL4* hh^{IJ}/hh^{IJ} embryos. *wg* expression is now detected in the alternating parasegments and naked cuticle is made in alternate segments. For the embryos stained with *wg* RNA (A,C,E,G), anterior is left and dorsal is up. For the cuticle preparations (B,D,F,H), anterior is left and ventral is forward.

Exogenous expression of CI(wt) in a PKA mutant background results in an increase of *wg* expression

It has been shown that PKA has a dual effect on the *hh* signaling pathway in vivo (Ohlmeyer and Kalderon, 1997) and has both a positive and negative effect on CI activity in cell culture (Chen et al., 1998**).** We have demonstrated that CI is the target for the negative regulation of PKA; however, the target of the positive PKA effect is unknown. To determine whether PKA could modulate the CI(m1-4)-mediated stimulation of *wg* expression in vivo, we crossed homozygous *UAS-HACI (wt or m1-4)*; *UAS-R** flies to *prd-GAL4*/*MKRS* flies and generated embryos that express HACI (wt or m1-4) and the mutant PKA regulatory subunit, R*, in the *prd* domains. The *UAS-R** transgene encodes a mutant form of the PKA regulatory subunit that inhibits PKA activity in imaginal discs and embryos by binding to the catalytic PKA subunit constitutively (Li et al., 1995). We expected that, if the small, positive regulation of CI by PKA was required in addition to the PKA-negative effect, the enhanced *wg* expression seen in the *UAS-HACI(m1-4)/+; prd-GAL4/+* embryos might be suppressed by the dominant negative PKA regulatory subunit. As shown in Fig. 6, expression of the dominant PKA mutant

Fig. 6. Suppression of PKA activity allows the HACI(wt) protein to increase *wg* expression but has no effect on the activity of HACI(m1-4). (A) The *wg* expression pattern of *UAS-R*/prd-GAL4* embryos. Expanded *wg* expression is seen in the 4th and 6th wg stripe. (B) The *wg* expression pattern of *UAS-HACI(wt)/+; UAS-R*/prd-GAL4* embryos. When HACI(wt) and R* are expressed together in the *prd* stripes, *wg* expression is broadened. Without R*, HACI(wt) is unable to increase *wg* expression(Fig. 4C). (C) The *wg* expression pattern of *UAS-HACI(m1-4)/+; UAS-R*/prd-GAL4* embryos. The *wg* expression pattern is indistinguishable from that of *UAS-HACI(wt)/+; UAS-R*/prd-GAL4* and *UAS-HACI(m1-4)/+; prd-GAL4/+* embryos. Anterior is left and ventral is forward. In B, anterior is left and dorsal is up.

in the *prd* domain causes mild *wg* overexpression in only 6.3% of the embryos (30/479 embryos counted), assuming that 50% of the embryos express the GAL4 activator (Fig. 6A). These results are in agreement with those reported by Ohlmeyer and Kalderon (1997). When CI(wt) is ectopically expressed in the *prd* domains, 40% of the embryos (288/728 embryos) overexpress the *wg* message (Fig. 6B). 50% of the *UAS-HACI(m1-4)/+; UAS-R*/prd-GAL4* embryos (341/674 embryos) overexpress *wg* (Fig. 6C). Therefore, the inhibition of PKA activity sensitizes the system so that exogenously expressed CI(wt) is able to induce ectopic *wg* expression, while CI(wt) cannot when it is overexpressed in the *prd-GAL4* genetic background. We could not detect a decrease in *wg* expression in *UASHACI(m1-4)/+; UAS-R*/prd-GAL4* embryos compared to *UAS-HACI(m1-4)/+; prd-GAL4/+* embryos.

DISCUSSION

A mutation of any one of the three PKA sites in CI is sufficient to abolish proteolysis of CI and increase CI transcriptional activity in cell culture

As shown in Fig. 1, the PKA sites at Ser-838, Ser-856 and Ser-892 in CI must remain intact for the regulation of CI proteolysis and transcriptional activity. Mutating any of the sites to alanine inhibits the proteolysis of CI leading to a 10-fold increase in CI activity. Furthermore, two-dimensional tryptic peptide maps show that PKA can specifically phosphorylate these sites in vitro; with the excess kinase present in the in vitro assays, these sites are not phosphorylated when the serines are mutated to alanines. These results support the hypothesis that the RRxS/T PKA sites are directly phosphorylated by PKA in vivo as well. In addition, all three sites in CI must be phosphorylated for the processing of CI to a repressor of *hh* target genes. These sites must also be intact for HH regulation. In Cl-8 or Kc cells, the activity of CI(m1-4) is unaffected by the addition of a HH signal while the wild-type CI activity is increased 6- to 10-fold when the cells are stimulated by HH. CI appears to be much more active in Cl-8 than Kc cells. One explanation for this observation is that the *hh* signaling cascade is more active in the disc-derived Cl-8 than embryo-derived Kc cells. In our model, the PKA phosphorylation of CI would ensure the processing of CI to the repressor form of the protein in cells that do not receive a HH signal. When HH reaches the cell surface, we hypothesize that it affects the ability of CI to be phosphorylated. Because the genetic data suggests that *hh* does not regulate PKA directly (Jiang and Struhl, 1995; Li et al., 1995; Ohlmeyer and Kalderon, 1997), it may be that *hh* affects the phosphorylation state of CI by activating a phosphatase, or through changing the accessibility of CI to a phosphatase. In support of this idea is the observation that the phosphatase inhibitor, okadaic acid, stimulates CI proteolysis, even in the presence of a HH signal (Y. C., unpublished data). HH signaling stimulates *fu* kinase activity to transform full-length CI to a transcriptional activator (Ohlmeyer and Kalderon, 1998). It may also be that *fu* activity renders full-length CI inaccessible to PKA phosphorylation.

We observed that the mutation of the Thr-1006 PKA site, RRQT, does not have any effect on CI function. This is expected from the work of Prorok and Lawrence (1989) who showed that peptides with threonines at the phosphorylation position are poor substrates for PKA activity.

Overexpression of CI(m1-4) increases *wg* expression and suppresses the *hh* mutant phenotype in developing embryos

We have also examined the activity of CI(wt) and CI(m1-4) in the developing embryo and find that CI(m1-4) can activate *hh* target gene expression even in the absence of *hh* signaling. When overexpressed in the *prd* domain, CI(wt) is neither sufficient to increase *wg* expression nor to suppress the *hh* mutant phenotype. This result differs from that presented by Alexandre et al. (1996), who demonstrated that overexpression of wild-type CI in the *engrailed* (*en*) domain increases the *wg* message and, in the *hairy* (*h*) domain, can suppress the mutant *hh* phenotype. This discrepancy could be due to differences among the *en-GAL4*, *h-GAL4* and *prd-GAL4* lines used in these experiments, although we do not observe any increase in *wg* expression when CI(wt) is expressed in the *h* domain by the *h-GAL4* (Brand and Perrimon, 1993) insertion (Chen and Smolik, unpublished observation). When expressed in the *prd* domain, CI(wt) can overexpress *wg* in a small percentage (7%) of the embryos. However, both the percentage and the degree of *wg* overexpression are significantly smaller and weaker when compared to embryos that overexpress CI(m1-4).

Ectopic expression of CI(m1-4) in the *prd* domain causes the expression of *wg* to expand into the anterior portion of the *prd* domain. This region includes cells that receive a HH signal as well as those that do not. By analogy to what occurs in discs (Aza-Blanc et al., 1997), it is hypothesized that, in the anterior cells that do not receive a HH signal, endogenous CI is proteolysed to a repressor of *wg* expression. CI(m1-4) is able to activate *wg* expression in these cells suggesting that, as in cell culture, it is not a substrate for proteolysis.

The phenotype that results from the CI(m1-4) overexpression is a loss of denticles in the anterior and posterior rows of the ventral denticle belts. It is known that the formation of naked cuticle absolutely requires *wg* activity (Bejsovec and Wieschaus, 1993). Thus the elevated levels of *wg* in cells that normally express *wg* at lower levels can account for the posterior row cells variably assuming a naked-cuticle cell fate. However, the loss of the anterior row is harder to explain. EN-expressing cells that abut the A/P boundary form naked cuticle and the row of cells just posterior to this also express EN and produce the anterior row denticles (Bejsovec and Wieschaus, 1993). These denticles absolutely require EN activity and are dependent on *wg* as well. Perhaps the increase in *wg* along the A/P boundary affects the levels or activity of EN so that the anterior row cells take on a more anterior fate.

While the CI(m1-4) embryos have a phenotype consistent with the overexpression of *wg*, they do not exhibit the loss of entire denticle belts that is seen when *wg* is overexpressed in the *h* domain (Wilder and Perrimon, 1995) or when ubiquitously expressed by a heat-shock (HS) promoter (Noordermeer et al., 1992). One likely explanation for this result is that the overexpression of *wg* that is mediated by CI is not as extreme as the overexpression of *wg* by the HS promoter or by GAL4. An alternate hypothesis is that the constitutive activity of CI(m1-4) causes the overexpression of more than one target gene and the relative dosages of these gene products modulate the activity of high levels of *wg*.

As shown in Fig. 5, CI(m1-4) can rescue the *hh* mutant phenotype when expressed in the *prd* domain. Together with the cell culture results, this observation suggests that a CI protein that is independent of the negative regulation of PKA, can bypass the requirement for a HH signal. The simplest explanation for the differential effect of CI(wt) and CI(m1-4) on *wg* expression is that CI(wt) is still subject to *hh* regulation and proteolytic processing within the *prd* domain, while CI(m1-4) is no longer a target for proteolysis and becomes constitutively active even in cells that do not receive a HH signal.

The recent work of Methot and Basler (1999) suggests that this interpretation is too simplistic. They demonstrated that the suppression of CI proteolysis may be necessary but not sufficient to by-pass the HH regulation of CI activity. In wing discs, a mutant CI that cannot be proteolyzed is unable to activate *hh* target-gene expression in the absence of a HH signal. These mutant transgenes contain the consensus RRxS/T PKA sites. Taken together with these results, our data suggest that the PKA phosphorylation of these sites is important for (1) regulating the proteolysis of CI and (2) for modulating the HH regulation of CI activity. While the dephosphorylation of one PKA site is sufficient to protect CI from proteolysis, the dephosphorylation of more than one site may be required to by-pass the HH signal.

Exogenously expressed wild-type CI can increase *wg* expression if endogenous PKA activity is suppressed

Cell culture studies demonstrated that, in addition to the negative regulation of CI, PKA could activate gene expression in a CI-dependent manner (Chen et al., 1998). Genetic studies have suggested that PKA can activate *hh* target genes in a *ci*-dependent but CI-level-independent fashion (Ohlmeyer and Kalderon, 1997). We wished to determine whether we could detect the CI-dependent PKA activation of *wg* expression in vivo. We reasoned that if PKA activated the full-length activator form of CI then CI(m1-4) activity could be suppressed through the inhibition of PKA in developing embryos. Although the activation in cell culture was insignificant (2-fold), it was possible that it represented a discernible activity in vivo. We expressed CI(wt) and CI(m1-4) together with a dominant negative PKA regulatory subunit (R*) in the *prd* domain. We expected that the increase of *wg* in the *UAS-HACI(m1-4)/+; UAS-R*/prd-GAL4* embryos would be suppressed, but this was not the case. The *wg* expression pattern in these animals is indistinguishable from that found in the *UAS-HACI(m1-4)/+; prd-GAL4/+* embryos.

In the absence of *hh* and PKA signaling, *wg* expression is undetectable although the full-length CI levels are increased (Ohlmeyer and Kalderon, 1997). This result suggests that *wg* expression can be uncoupled from the increase in full-length CI levels. We find that exogenously expressed CI(wt) can activate *wg* expression in the absence of *hh* and PKA signaling. A possible explanation for this difference is that, in our experiments, the higher levels of exogenous CI(wt) activated in response to the inhibition of PKA activity is sufficient to activate *wg* but the endogenous levels of wild-type CI protein are not. It is of interest that the dose of exogenous CI(wt) expressed by *prd-Gal4* is not sufficient to activate *wg* expression unless the levels of PKA activity are decreased. In this case, the increased dosage of CI that is not phosphorylated by PKA may be sufficient to overcome the antagonistic effects of *Su(fu)* and allow a threshold amount of activated CI to accumulate.

This work was supported by a grant from the National Institutes of Health (DK44239) and fellowships from the Swiss National Science Foundation and the Société Académique Vaudoise (to J.-R. C.)

REFERENCES

Alcedo, J., Ayzenzon, M., Ohlen, T. V., Noll, M. and Hooper, J. E. (1996). The *Drosophila smoothened* gene encodes a seven-pass membrane protein, a putative receptor for the *hedgehog* signal. *Cell* **86,** 221-232.

Alexandre, C., Jacinto, A. and Ingham, P. W. (1996). Transcriptional activation of *hedgehog* target genes in *Drosophila* is mediated directly by the cubitus interruptus protein, a member of the GLI family of zinc finger DNA-binding proteins. *Genes Dev.* **10,** 2003-2013.

Aza-Blanc, P., Ramirez-Weber, F.-A. and Kornberg, T. B. (1997). Proteolysis that is inhibited by hedgehog targets cubitus interruptus protein to the nucleus and converts it to a repressor. *Cell* **89,** 1043-1053.

Bejsovec, A. and Wieschaus, E. (1993). Segment polarity gene interactions modulate epidermal patterning in *Drosophila* embryos. *Development* **119,** 501-517.

Boyle, W.J., van der Geer, P. and Hunter, T. (1991). Phosphopeptide mapping and phosphoaminoacid analysis by two-dimensional separation on thin-layer cellulose plates. *Methods Enzymol.* **201**, 110-149

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118,** 401-415.

Chen, Y., Gallaher, N., Goodman, R. H. and Smolik, S. M. (1998). Protein kinase A directly regulates the activity and proteolysis of *cubitus interruptus*. *Proc. Natl. Acad. Sci. USA* **95,** 2349-2354.

Currie, D. A., Milner, M. J. and Evans, C. W. (1988). The growth and differentiation in vitro leg and wing imaginal disc cells from *Drosophila melanogaster*. *Development* **102,** 805-814.

DiNardo, S., Sher, E., Heemskerk-Jongens, J., Kassis, J. A. and O'Farrell, P. H. (1988). Two-tiered regulation of spatially patterned *engrailed* gene expression during *Drosophila* embryogenesis. *Nature* **332,** 604-609.

Dominguez, M., Brunner, M., Hafen, E. and Basler, K. (1996). Sending and receiving the *hedgehog* signal: control by the *Drosophila* Gli protein cubitus interruptus. *Science* **272,** 1621-1625.

Drosophila embryo. *Development* **116,** 711-719.

Fleming, R. J., Scottgale, T. N., Diederich, R. J. and Artavanis-Tsakonis, S. (1990). The gene *Serrate* encodes a putative FGF-like transmembrane protein essential for proper ectodermal development in *Drosophila melanogaster*. *Genes Dev.* **4,** 2188-2201.

Forbes, A. J., Nakano, Y., Taylor, A. M. and Ingham, P. W. (1993). Genetic analysis of *hedgehog* signaling in the *Drosophila* embryo. *Development* **1993 Supplement,** 115-124.

Heuvel, M. v. d. and Ingham, P. W. (1996). *smoothened* encodes a receptor-like serpentine protein required for *hedgehog* signaling. *Nature* **382,** 547-551.

Ingham, P. W. (1993). Localized *hedgehog* activity controls spatial limits of *wingless* transcription in the *Drosophila* embryo. *Nature* **366,** 560-562.

Ingham, P. W. and Hidalgo. (1993).Regulation of wingless transcription in the Drosphila embryo. *Development* **117,** 283-291.

Jiang, J. and Struhl, G. (1995). Protein kinase A and *hedgehog* signaling in *Drosophila* limb development. *Cell* **80,** 563-572.

Johnson, R. L., Grenier, J. K. and Scott, M. P. (1995). *Patched* overexpression alters wing disc size and pattern: transcriptional and post-transcriptional effects on *hedgehog* targets. *Development* **121,** 4161-4170.

Kennelly, P. J. and Krebs, E. G. (1991) Consensus sequences as substrate specificity determinants for protein kinases and protein phosphatases. *J. Biol. Chem*. **266,** 15555-15558.

Lee, J. J., Kessler, D. P. V., Parks, S. and Beachy, P. A. (1992). Secretion and localization suggest a role in positional signaling for products of the segmentation gene *hedgehog*. *Cell* **71,** 33-50.

Lepage, T., Cohen, S. M., Diaz-Benjumea, F. J. and Parkhurst, S. M. (1995). Signal transduction by cAMP-dependent protein kinase A in *Drosophila* limb patterning. *Nature* **373,** 711-715.

Lewis, E. B. (1978). A gene complex controlling segmentation in *Drosophila*. *Nature* **276,** 565-570.

Li, W., Ohlmeyer, J. T., Lane, M. E. and Kalderon, D. (1995). Function of protein kinase A in *hedgehog* signal transduction and *Drosophila* imaginal disc development. *Cell* **80,** 553-562.

Lindsley, D. L. and Zimm, G. G. (1992). *The Genome of Drosophila melanogaster.* San Diego, CA: Academic Press.

Marigo, V., Davey, R. A., Zuo, Y., Cunningham, J. M. and Tabin, C. J. (1996). Biochemical evidence that *patched* is the hedgehog receptor. *Nature* **384,** 176-179.

Martinez Arias, A., Baker, N. E. and Ingham, P. W. (1988). Role of segment polarity genes in the definition and maintenance of cell states in the *Drosophila* embryo. *Development* **103,** 157-170.

Methot, N and Basler, K. (1999). Hedgehog controls limb development by regulating the activities of distinct transcriptional activator and repressor forms of Cubitus interruptus. *Cell* **96,** 819-831.

Motzny, C. K. and Holmgren, R. (1995). The Drosophila *cubitus interruptus* protein and its role in the *wingless* and *hedgehog* signal transduction pathways. *Mech. Dev.* **52,** 137-150.

Noordermeer, J., Johnston, P., Rijsewijk, F., Nusse, R. and Lawrence, P. A. (1992). The consequences of ubiquitous expression of the *wingless* gene in the

Ohlen, T. v., Lessing, D., Nusse, R. and Hooper, J. E. (1997). *Hedgehog* signaling regulates transcription through cubitus interruptus, a sequence-specific DNA binding protein. *Proc. Natl. Acad. Sci. USA* **94,** 2404-2409.

Ohlmeyer, J. T. and Kalderon, D. (1997). Dual pathways for induction of *wingless* expression by protein kinase A and hedgehog in *Drosophila* embryos. *Genes Dev.* **11,** 2250-2258.

Ohlmeyer, J. T. and Kalderon, D. (1998). Hedgehog stimulates maturation of Cubitus interruptus into a labile transcriptional activator. *Nature* **396,** 749-753

Pan, D. and Rubin, G. M. (1995). cAMP-dependent protein kinase and *hedgehog* act antagonistically in regulating *decapentaplegic* transcription in *Drosophila* imaginal disc. *Cell* **80,** 543-552.

Pearson, R. B. and Kemp, B. E. (1991). Protein kinase phosphorylation site sequences and consensus specificity motifs: Tabulations. In *Methods in Enzymology*, V.200, pp 62-77. New York: Academic Press, Inc.

Prorok, M. and Lawrence, D. S. (1989). Intrasubstrate steric interactions in the active site control the specificity of the cAMP-dependent protein kinase. *Biochem. Biophys. Res. Com.* **158,** 136-140.

Robbins, D. J., Nybakken, K. E., Kobayashi, R., Sisson, J. C., Bishop, J. M. and Therond, P. P. (1997). Hedgehog elicits signal transduction by means of a large complex containing the kinesin-related protein costal2. *Cell* **90,** 225-234.

Rose, R. E., Gallaher, N. M., Andrew, D. J., Goodman, R. H. and Smolik, S. M. (1997). The CRE binding protein dCREB-A is required for *Drosophila* embryonic development. *Genetics* **146,** 595-606.

Sisson, J. C., Ho, K. S., Suyama, K. and Scott, M. P. (1997). *Costal2*, a novel kinesin-related protein in the *hedgehog* signaling pathway. *Cell* **90,** 235-245.

Slusarski, D. C., Motzny, C. K. and Holmgren, R. (1995). Mutations that later the timing and pattern of *cubitus interruptus* gene expression in *Drosophila melanogaster*. *Genetics* **139,** 229-240.

Spradling, A. (1986). P element-mediated transformation. In *Drosophila, a Practical Approach "* (ed. D. B. Roberts), pp. 175-198. New York: IRL Press.

Tabata, T., Eaton, S. and Kornberg, T. B. (1992). The *Drosophila hedgehog* gene is expressed specifically in posterior compartment cells and is a target of *engrailed* regulation. *Genes Dev.* **6,** 2635-2645.

Tautz, D. and Pfeile, C. (1989). A non-radioactive *in situ* hybridization method for the localization of specific RNAs in the *Drosophila* embryo reveals translational control of the segmentation gene hunchback. *Chromosoma* **98,** 81-85.

Therond, A. P., Knight, J. D., Kornberg, T. B. and Bishop, J. M. (1996). Phosphorylation of the fused protein kinase in response to signaling from *hedgehog*. *Proc. Natl. Acad. Sci. USA* **93,** 4224-4228.

van Leeuwen, F., Samos, C. H. and Nusse, R. (1994). Biological activity of soluble wingless protein in cultured Drosophila imaginal disc cells. *Nature* **368,** 342-344.

Wilder, E. L. and Perrimon, N. (1995). Dual functions of *wingless* in the *Drosophila* leg imaginal disc. *Development* **121,** 477-488.

Yoffe, K. B., Manoukian, A. S., Wilder, E. L., Brand, A. H. and Perrimon, N. (1995). Evidence for *engrailed*-independent *wingless* autoregulation in Drosophila. *Dev. Biol.* **170,** 636-650.