Transcriptional regulation of the Hedgehog effector CI by the zinc-finger gene combgap

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SUMMARY

Members of the Hedgehog (HH) family of secreted signaling molecules specify cell fate during animal development by controlling the activity of members of the Gli family of zinc-finger transcription factors in responding cells. In Drosophila the Gli homolog, cubitus interruptus (CI), is expressed only in the anterior compartment where it represses targets such as the signaling molecule genes decapentaplegic (dpp) and wingless (wg). HH is expressed in the posterior and diffuses into the anterior where it antagonizes CI repression resulting in dpp and wg expression immediately anterior to the compartment border. Reducing CI levels results in misexpression of wg and dpp, while CI misexpression in the posterior disrupts differentiation. Thus, normal disc patterning requires high levels of CI in the anterior and the absence of CI in the posterior. Here we show that mutations in combgap (cg) result in deregulation of CI expression, which is now expressed at much lower levels and ubiquitously, i.e., also in the posterior. Consequently, cg mutants phenocopy ci loss-of-function mutants in the anterior and ci gain-of-function mutants in the posterior. cg encodes a putative DNA-binding protein that regulates both transcriptional activation and repression of the ci gene.

Key words: Hedgehog, Cubitus interruptus, Gli, Zinc finger, Combgap, Imaginal discs, Drosophila

INTRODUCTION

Secreted polypeptide signaling molecules belonging to several different families regulate the specification of different cell fates in many animal systems. The Hedgehog (HH) family includes some of the most important regulators of pattern formation (Hammerschmidt et al., 1997; Ingham, 1994). Members control segmental patterning in Drosophila embryos (Mohler, 1988), specification of the anteroposterior axis in both vertebrate and insect limbs (Basler and Struhl, 1994; Riddle et al., 1993) and specification of the dorsoventral axis in the vertebrate neural tube (Briscoe and Ericson, 1999). Within these systems, hh genes are expressed in discrete domains and secreted HH proteins subsequently regulate patterning by acting as morphogens that induce expression of different target genes above distinct threshold concentrations or by activating expression of additional signaling molecules that in turn act as morphogens (Basler and Struhl, 1994; Briscoe and Ericson, 1999; Strigini and Cohen, 1997).

Activation of the HH signaling pathway in responding cells regulates gene expression by modulating the activity of the Gli family of zinc-finger transcription factors, including cubitus interruptus (CI) in Drosophila (Ruiz i Altaba, 1999). The primary function of HH signaling in Drosophila appears to be to antagonize the constitutive phosphorylation of CI by protein kinase A (PKA; Chen et al., 1999b; Wang et al., 1999). Full-length CI (CI-155) can act as a transcriptional activator but the fully phosphorylated form is impeded from entering the nucleus and it is targeted for proteolytic cleavage to a truncated form (CI-75), which acts as a transcriptional repressor (reviewed in Aza-Blanc and Kornberg, 1999; Chen et al., 1999a). Thus, HH signaling can activate gene expression directly by raising the level of the activator, CI-155, or more indirectly by reducing the level of the repressor, CI-75, relieving the repression of other genes.

Correct spatial patterning, thus, requires the modulation of CI activity in the appropriate places and this involves the restriction of both hh and ci expression in developing tissues. The embryonic segments and imaginal discs (the larval tissue giving rise to much of the adult including the appendages) of Drosophila are divided into two lineage units, the anterior and posterior compartments (Crick and Lawrence, 1975) which are characterized by the expression of the related homeobox genes engrailed (en) and invected (inv) in the posterior and ci in the anterior (Blair, 1995; Eaton and Kornberg, 1990; Kornberg et al., 1985; Orenic et al., 1990; Tabata et al., 1995). There is no ci expression in the posterior because it is directly repressed by EN (Eaton and Kornberg, 1990; Schwartz et al., 1995), whereas CI plays no role in regulating en expression. The posterior compartment is also characterized by the expression of hh; it is repressed in the anterior by CI (Dominguez et al., 1996). The segments and discs are thus divided into a CI-positive anterior and a EN/HH-positive posterior.
HH regulates the expression of several genes, including those encoding the secreted signaling molecules wingless (wg, a WNT, in the embryonic segment and ventral leg disc) and decapentaplegic (dpp, a TGFβ, in the wing disc and the dorsal leg; Basler and Struhl, 1994; Capdevila and Guerrero, 1994; Ingham et al., 1991). These targets are repressed in the posterior by EN (Sanicola et al., 1995; Tabata et al., 1995; Zecca et al., 1995), but HH protein diffusing from the posterior induces their expression in a stripe immediately anterior to the compartment border, the width of the stripe revealing the extent to which HH can diffuse into the anterior. HH signaling in this stripe of cells antagonizes the phosphorylation and degradation of CI, resulting in high levels of the activator form, CI-155 and low levels of the repressor, CI-75 form at the compartment border, whereas outside of this stripe CI-75 is the prevalent form (Aza-Blanc et al., 1997). dpp expression in the wing is both activated and repressed by CI away from the compartment border dpp is repressed by CI-75 and, in fact, loss of ci anywhere in the anterior results in misexpression of dpp (Dominguez et al., 1996), which demonstrates that merely reducing the level of CI-75 is sufficient to allow dpp expression. HH signaling at the compartment border reduces the level of CI-75 and although this is sufficient for dpp expression, it appears that activation of expression by CI-155, stabilized at the compartment border, is required to achieve the normal levels of expression found in wild-type wings (Methot and Basler, 1999). The mechanism of CI regulation of wg expression in the embryo and ventral leg is less well understood, because, although it appears that CI can activate wg expression (Alexandre et al., 1996; Chen et al., 1999b), it is unclear whether it also is required to repress its expression away from the compartment border.

Gain and loss of HH activity results in characteristic phenotypes in imaginal discs. Ubiquitous expression of HH has no effect in the posterior compartment of leg and wing discs; in the anterior, it results in misexpression of dpp in all anterior cells of the wing and dorsal leg discs and of wg in all the cells of the ventral leg (Basler and Struhl, 1994). This results in characteristic overgrowth phenotypes in both the wing and the leg that can be directly related to the misexpression of dpp and wg (Campbell and Tomlinson, 1995; Diaz-Benjumea et al., 1994). Loss of HH activity results in the loss of dpp or wg expression, and both leg and wing discs fail to grow, again a result that is directly related to the loss of wg and dpp (Basler and Struhl, 1994). Proteins involved in transducing the HH signal (Murone et al., 1999) can be divided into those required for proteolysis of CI, such as patched (PTC; Ingham, 1998), and those required for antagonizing this and for maintaining the unphosphorylated activator form of CI, such as smoothened (SMO; Alcedo et al., 1996; van den Heuvel and Ingham, 1996); mutations in genes encoding these proteins phenocopy HH gain-of-function and HH loss-of-function, respectively.

Loss of CI also phenocopies HH gain-of-function in the wing because, as discussed above, CI is required to repress dpp expression in most of the anterior compartment. Misexpression of CI to very high levels in the anterior can also phenocopy HH gain-of-function (Hepker et al., 1997), presumably because this results in high levels of the full-length form and sufficient amounts can gain access to the nucleus (Chen et al., 1999a). Misexpression of CI in the posterior can disrupt normal differentiation in the wing and is associated with defects in wing-vein patterning (Hepker et al., 1997). In fact, many of the original ci mutants are gain-of-function alleles in which ci is expressed in the posterior as well as the anterior; these mutants have characteristic gaps in wing-vein IV (Slusarski et al., 1995). Thus, normal disc patterning requires CI expression to be restricted to the anterior compartment and for CI protein activity to be modulated at the compartment boundary.

Here, we describe a molecular and genetic characterization of the combgap (cg) gene and show that mutations in this gene result in a hedgehog gain-of-function phenotype in the anterior of the leg. However, unlike other mutants with this phenotype, such as ptc, cg mutants also disrupt patterning in the posterior of the wing in a similar fashion to ectopic ci expression. We show that both anterior and posterior compartment phenotypes in cg mutants are due to deregulation of ci expression: instead of just being expressed at high levels in the anterior compartment, ci is expressed at uniform low levels throughout the disc, i.e., also in the posterior. The hedgehog gain-of-function phenotype appears to be the direct result of the lower levels of CI not being sufficient to repress dpp and wg expression. The patterning defect in the posterior is also identical to that produced by direct misexpression of ci. cg encodes a putative transcription factor that, thus, appears to be required for normal regulation of transcription of the ci gene.

**MATERIALS AND METHODS**

**P-element screen, fly stocks, mutant analysis and clonal analysis**

A collection of P-element insertion lines generated by the Berkeley Drosophila Genome Project (BDGP) and maintained by the Bloomington stock center was screened for interesting lacZ expression patterns in the leg and wing imaginal discs; line l(2)07659 was selected for further study based on its homozygous phenotype rather than its lacZ expression pattern. This line was maintained over the SM6a-TM6B balancer and homozygous larvae were selected by the absence of the Tb marker. Fly stocks dpp-lacZ (dpp106,8), wg-lacZ (wg265), hh-lacZ (hh42), hh42 arm-lacZ/ FRT 42, cg-hh ts, ap-Gal4 (apGal4F4), UAS-ci (UAS-ci/UAS-ci) and FRT 42 are described in detail at Flybase (http://flybase.bio.indiana.edu). The ciP element is unpublished and was identified fortuitously in another mutant stock.

It is homozygous viable with wing duplications in a small percentage of flies and enlarged antennae in most flies. It is pupal lethal over ci07, ciD or a deficiency, with larvae having leg and wing discs showing a hedgehog gain-of-function phenotype. Molecular analyses reveal a 6 kb insertion upstream of the transcription start site (not shown). cg clonal analysis was performed using larvae of the genotype hsflp; FRT 42 arm-lacZ/FRT 42 cg07659, as described previously (Campbell and Tomlinson, 1998). The cg hh mutant expression was performed with a catalytically inactive form of the hh gene. hh07658, hh07658/S64a-TM6B stock; the larvae were raised until early second instars at 17°C and then shifted to 31°C until late thirds, when their discs were dissected and fixed. The ci hh mutant analysis was performed in a similar fashion by crossing hh07658/TM6B; ci07658/TM6B; ciP/+. **Gene expression studies**

X-gal stains, immunohistochemistry, immunofluorescence and in situ analyses were performed as previously described (Campbell and Tomlinson, 1998). Antibodies used are to AL (rat, diluted 1:1000; Campbell and Tomlinson, 1998), β-gal (Capell, rabbit, diluted 1:2000), CI (AbN; rabbit, diluted 1:250; this recognizes both the full-
length and degraded form; Aza-Blanc et al., 1997), CI (2A1; rat monoclonal, supernatant diluted 1:1; this recognizes only the full-length form; Slusarski et al., 1995). A riboprobe was generated from the X27 cDNA for use in the in situ analysis.

**Generation of new cg mutants**

Initially small deletions were made by excision of the P-element using the Δ2-3 transposase source, yielding several lines with phenotypes equivalent to or less severe than cg07659. Some deletions, such as that in Df(2R)cg315 extended 3’ to the insertion and removed at least one transcription initiation start site and also the adjacent transcription unit. Others, such as the deletion in cg262, extended 5’ to the insertion (in this case extending almost to the start of the X31 cDNA but not into the coding region). Df(2R)cg315 is embryonic lethal and is not rescued by CG247, indicating that it removes more than the cg gene, but it has an identical phenotype over both cg07659 and cg262 as cg/07659 homozygotes, cg262 is rescued by CG247 and homozygotes also have an identical phenotype to cg07659. However, molecular analyses of several hundred deletions revealed that none extended into the protein-coding region of the cg gene so they could not be classified as nulls. Because the cg/07659 line shows lacZ expression, the analysis of dpp and wg expression was carried out using cg262/Df(2R)cg315 larvae.

EMS mutants were generated using standard techniques. The initial mutant screen was based on the observation that en x/+ cg transheterozygotes have a gap in vein IV in a high percentage of flies. Wild-type flies were mutagenized with EMS and crossed to en59 and approximately 10,000 en59 heterozygotes were screened for gaps in vein IV. This screen yielded a single new cg allele, cg14, based on its inability to complement cg07659 and a substitution of a conserved amino acid in the third zinc finger. An additional screen was performed to revert the dominant negative activity of cg14, cg14 heterozygotes were mutagenized and crossed to en59 heterozygotes, and about 10,000 en59 x/+ cg14 transheterozygotes were screened for flies with a reduced gap or no gap in vein IV. A single putative null cg mutant, cg432, was identified in this screen. To determine the molecular basis of these two mutants, genomic DNA was isolated from homozygous larvae and specific primers were used to amplify the cg coding region, which was then sequenced.

**Rescue of ci and cg mutants by UAS-ci**

For rescue of ci mutants by UAS-ci, ap-Gal4/+; ci041/+; flies were crossed to dpp-lacZ/+; UAS-ci/+; ap-Gal4 and the discs from the resulting larvae were fixed and stained with X-gal and anti-Ci. ap-Gal4/dpp-lacZ/UAS-ci+; ci041/+ discs were identified by overgrowth and by high levels of CI in the dorsal compartment of the wing. For rescue of cg mutants by ci, dpp-lacZ cg352; UAS-ci/SM6a-TM6B flies were crossed to Df(2R)cg315; C765/SM6a-TM6B. Leg discs from the non-Th larvae were stained with X-gal. Gal4 is expressed ubiquitously in C765.

**Cloning of cg and molecular biology**

Genomic DNA 5’ to the P-element in cg07659 was cloned by plasmid rescue and used to screen a lambda genomic library; approximately 50 kb of genomic DNA was isolated. This was used to screen cDNA libraries identifying two transcripts close to the insertion site. Further analysis revealed the P-element to be inserted in an intron in the 5’ UTR of one of these transcripts, indicating this was most likely to correspond to cg. Over 80 cDNAs were pulled out and the two longest, X27 and X31, were sequenced. This sequence and partial characterization of the other cDNAs by restriction analysis revealed several alternative transcripts, but that almost all of the variation existed outside of the coding region apart from an alternative C terminus (Fig. 5). An 11.5 kb XbaI genomic fragment including all of this transcript was cloned into pW8 and used to transform flies (CG247). This fragment minus a central 3.4 StuI fragment was also cloned into pW8 and used to transform flies (CG248).

**RESULTS**

**P-element mutant line, l(2)07659, phenocopies HH gain-of-function**

Ubiquitous expression of hh in the leg imaginal discs of Drosophila results in a transformation of the normal circular disc into an anteriorly elongated oval (Campbell and Tomlinson, 1995). This is associated with misexpression of dpp in the dorsal anterior and of wg in the ventral anterior. The Wg/DPP target, aristless (al), is expressed as a line at the interface between these two domains. In wild-type discs, there is only a spot of al expression in the center (Campbell and Tomlinson, 1995). Screening through a collection of P-element-induced mutations generated by the BDGP identified a line, l(2)07659, in which homozygous larvae possessed leg discs morphologically identical to those generated by ubiquitous hh expression (Fig. 1B). This phenotype was not associated with ectopic expression of hh, and loss of HH activity using a temperature-sensitive mutant had no effect on the phenotype of l(2)07659 discs (Fig. 1C,D). However, both dpp and wg were misexpressed in the anterior of these discs, accounting for the overgrowth and ectopic al expression (Fig. 1F). In the posterior, wg and dpp were repressed as in wild-type discs. Curiously, although dpp was misexpressed, the level of expression, even at the compartment border, was lower than in wild-type discs (Fig. 1G,H). Ubiquitous expression of hh also induces ectopic dpp expression and overgrowth in the wing (Basler and Struhl, 1994), but there was no overgrowth in l(2)07659 mutant wing discs and there was only very weak ectopic dpp expression (Fig. 1L).

**l(2)07659 is an allele of cg and interacts with en and ci**

l(2)07659 was mapped to region 50E1-2 by the BDGP (Spradling et al., 1999). Mobilization of the P-element resulted in a high frequency of excisions (>50%) that were completely wild type, indicating that the P-element is responsible for the lethality of this line. Complementation tests with genes in the same region identified it as an allele of cg and it has been renamed cg07659. A single viable allele, cg1, had been identified previously with two main phenotypes: first, the number of sex comb teeth on the male first leg is increased (an anterior compartment phenotype associated with ectopic wg expression, Fig. 2E); and second, there is a gap in vein IV in the wing (a posterior compartment phenotype; Fig. 2B) (Waddington, 1953). cg107659 heterozygotes are also viable with a slightly more-severe phenotype than cg1 homozygotes (Fig. 2C,F). As described below, it appears that cg07659 is a very strong allele identical in severity to a putative null, cgA22.

Although cg07659 homozygotes phenocopy mutants such as ptc, Pka-C1 and costa (cos), which show hedgehog gain-of-function phenotypes, the posterior compartment in the latter mutants is unaffected, unlike cg adult wings, indicating that CG function may not be restricted to HH signal transduction. Previous studies showed that cg1 interacts strongly with en and some ci mutants (House, 1953, 1961; Waddington, 1953). The ci mutants used in these studies have subsequently been shown to be gain-of-function mutants in which ci is misexpressed in the posterior (Slusarski et al., 1995) resulting defects in posterior wing vein patterning, most notably loss
of vein IV. These genetic interactions were confirmed with cg<sup>07659</sup>, as follows. ci<sup>W</sup> is a weak dominant mutation with heterozygotes showing a variable loss of vein IV tissue (Fig. 2G). The cg<sup>07659</sup>; ci<sup>W</sup> transheterozygotes have a much stronger phenotype with vein IV being completely absent in all flies (Fig. 2H). Many viable en loss-of-function mutants have gaps in vein IV and some, for example en<sup>59</sup>, also show this phenotype very occasionally (<1%) as heterozygotes, but en<sup>59</sup> +/+ cg<sup>07659</sup> transheterozygotes showed this at a much higher frequency (25%; Fig. 2I). This suggested ci and possibly en activities may be disrupted in cg mutants.

**Cl expression is abnormal in cg mutant discs but posterior EN expression is unaffected**

Analysis of cg mutant discs revealed CI expression was abnormal. Instead of high levels in the anterior and no expression in the posterior, CI was expressed at low levels throughout (Fig. 3A-D). An antibody that recognizes all CI proteins (AbN; Aza-Blanc et al., 1997) revealed fairly uniform levels of expression in both anterior and posterior compartments of the leg and wing discs, but at much lower levels than are found in the anterior of wild-type discs. A CI antibody (2A1) that recognizes only the full-length form (stabilized by HH signaling; Slusarski et al., 1995) revealed
Loss of ci in the leg phenocopies HH gain-of-third instar wing discs (Fig. 3K-M). (defined by clonal analysis) do not express EN in late immediately anterior to the compartment border (Blair, 1992; Strigini and Cohen, 1997). the compartment boundary and this is HH-dependent en usually restricted to the posterior, in late third instars, anterior compartment of the wing. Although EN is had a minor effect on EN expression, but this was in the (Fig. 3H-J). and Kornberg, 1990), but posterior EN expression in cg mutant clones in the anterior showed autonomous gain of ci expression (Fig. 3H-J).

EN is required to repress ci in wild-type discs (Eaton and Kornberg, 1990), but posterior EN expression in cg mutant discs appeared normal (Fig. 3E-J). This was clearly demonstrated by clonal analysis as EN expression in cg mutant clones in the posterior was indistinguishable from that in surrounding wild-type cells (Fig. 3K-M). Thus, it appears that En is unable to repress ci expression normally in cg discs. Loss of cg had a minor effect on EN expression, but this was in the anterior compartment of the wing. Although EN is usually restricted to the posterior, in late third instars, en expression is activated in the anterior of the wing at the compartment boundary and this is HH-dependent (Blair, 1992; Strigini and Cohen, 1997). cg mutant cells immediately anterior to the compartment border (defined by clonal analysis) do not express EN in late third instar wing discs (Fig. 3K-M).

Loss of ci in the leg phenocopies HH gain-of-function

Loss of ci activity in the wing results in HH-independent dpp expression in the anterior compartment (Dominguez et al., 1996; Methot and Basler, 1999) so it appeared possible that the ectopic dpp and wg in the anterior of cg leg discs may result directly from the lowered CI levels found in these discs. Initially we investigated whether loss of ci in leg discs also resulted in a hedgehog gain-of-function phenotype as it does in the wing. This was achieved by analysis of hypomorphic ci loss-of-function allelic combinations ci<sup>n</sup>ci<sup>94</sup> or ci<sup>n</sup>ci<sup>D</sup>. These are pupal lethal with larvae having wing and leg discs that phenocopy ubiquitous hh expression (Fig. 4A,C,D). CI levels were dramatically reduced in these discs and the mutant phenotype was HH independent (Fig. 4B,F). dpp was misexpressed in the anterior wing pouch and in the dorsal anterior of the leg discs (Fig. 4C,D) and wg.

higher levels in the posterior than in most of the anterior. Clonal analysis was also used to demonstrate that the effect of cg on CI expression is cell autonomous. cg clones in the anterior showed autonomous reduction in ci levels whilst clones in the posterior showed autonomous gain of ci expression (Fig. 3H-J).

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was misexpressed in the ventral anterior of the legs (not shown). The ectopic dpp expression was repressed by raising ci levels with a wild-type UAS-ci transgene driven by a Gal4 line (Fig. 4E). Thus, normal dpp and wg expression requires high levels of CI in the anterior because this functions to repress their expression away from the compartment border.

**Raising CI levels in cg mutant leg discs suppresses the hedgehog gain-of-function phenotype**

To test whether the ectopic dpp and wg in cg leg discs is a direct result of the lowered ci levels in these discs, ci levels were raised in cg mutants with a ubiquitous Gal4 driver (Fig. 4G). The higher ci levels rescued the overgrowth and dpp misexpression phenotypes (Fig. 4H), indicating that these anterior compartment combgap phenotypes are the direct result of lowered ci levels and consequently that one of the functions of cg is to maintain high levels of ci expression in the anterior.

**cg encodes a ubiquitously expressed, putative transcription factor**

cg was cloned by plasmid rescue of the P-element in cg¹⁰⁷⁶⁵⁹ and subsequent screening of genomic and cDNA libraries. This P-element is inserted in an intron in the 5' UTR of a transcription unit that encodes for a protein consisting largely of ten C2H2 zinc fingers (and a defective eleventh finger, Fig. 5A-C). A genomic fragment, CG247, containing this unit can fully rescue the cg¹⁰⁷⁶⁵⁹ mutant while the same fragment containing a small deletion removing part of this zinc-finger gene can not (Fig. 5A), indicating that it corresponds to cg. cg is expressed ubiquitously at uniform levels in the imaginal discs (Fig. 5E,F), consistent with it being required in both anterior and posterior compartments.

**Generation of a putative null cg allele**

The molecular analysis cannot confirm that cg¹⁰⁷⁶⁵⁹ is a null allele so additional mutants were generated. Mobilization of the P-element in line cg¹⁰⁷⁶⁵⁹ generated several deletions (see Materials and Methods for more details) that had identical phenotypes to cg¹⁰⁷⁶⁵⁹ homozygotes. However, none of these deletions extended into the protein-coding region of the cg gene, so it was impossible to determine if they were complete loss-of-function alleles.

Consequently, an alternative approach was taken to generate point mutants, cg¹⁷⁴ and cg¹⁰²². cg¹⁷⁴ was identified because en⁵⁹+/+ cg¹⁷⁴ adults show a loss of vein IV (Fig. 6A). cg¹⁷⁴ is associated with a substitution of a conserved amino acid in the third zinc finger (Fig. 5D) and appears to be a dominant negative mutant because, although the vein IV phenotype over en⁵⁹ (Fig. 6A) is more penetrant than cg¹⁰⁷⁶⁵⁹ (100% compared with 25%), it has a weaker phenotype as a homozygote, showing no overgrowth in leg discs. In addition, cg¹⁷⁴ homozygotes have a weak dominant phenotype, having

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**Fig. 5.** Molecular biology of cg. (A) The cg genomic region showing the insertion site of the P-element in cg¹⁰⁷⁶⁵⁹, the location of cg cDNAs X27 and X31 (introns are not indicated) and an additional transcript T2. The 11.5kb fragment CG247 fully rescues cg mutants whilst the smaller fragment, CG248, lacking the 3.4kb Stu fragment, does not rescue. (B) cg encodes a protein containing ten C2H2 zinc fingers (and a defective eleventh finger, Fig. 5A-C). A genomic fragment, CG247, containing this unit can rescue the cg¹⁰⁷⁶⁵⁹ mutant while the same fragment containing a small deletion removing part of this zinc-finger gene can not (Fig. 5A), indicating that it corresponds to cg. cg is expressed ubiquitously at uniform levels in the imaginal discs (Fig. 5E,F), consistent with it being required in both anterior and posterior compartments.
venation defect in cg hypomorphs is very similar to that found in these ci mutants and it was shown that this phenotype is enhanced in cg/+; ci/+ transheterozygotes. These ci mutants, however, are gain-of-function mutants; they show ectopic expression of ci in the posterior (Slusarski et al., 1995). In fact, direct misexpression of ci in the posterior using the UAS/Gal4 system can also produce the same vein defects as seen in these mutants and in cg mutants (Hepker et al., 1997). Analysis of cg mutant discs reveals ectopic ci expression in the posterior (Fig. 3D), indicating that the cg posterior phenotype is almost certainly the direct result of deregulation of ci expression in this compartment.

CI expression is also abnormal in the anterior of cg mutant discs, being found at much lower levels than in wild-type discs. Loss of ci expression in the wing results in hedgehog gain-of-function phenotypes, including overgrowth and misexpression of dpp. Reduced CI levels in the leg also result in the characteristic overgrowth phenotype, with ectopic expression of wg and dpp, found following ubiquitous expression of HH – i.e., the same phenotype as that found in cg mutant leg discs. Support for the proposal that the anterior combgap phenotype in the leg is also the direct result of deregulation of ci expression, in this case lowered levels of expression, comes from the observation that raising ci levels in cg mutant leg discs using the UAS/Gal4 system can suppress the overgrowth and ectopic dpp expression.

One difference between ci and cg mutants is that wing discs from the former have a hedgehog gain-of-function phenotype with overgrowth and ectopic dpp in the anterior (Dominguez et al., 1996), whilst the latter do not show overgrowth and only very weak ectopic dpp. It is possible that the leg and wing are differentially sensitive to CI levels and the CI levels are still high enough in the wing in cg mutants to repress most dpp expression. Protein levels detected with antibody staining in ci hypomorphs and cg mutants are too low to detect significant differences with confidence, so the reason for the difference between ci and cg wings remains to be determined. CI is also required during embryogenesis, but the putative null cg mutant survives to the early pupal stage. This suggests either that lower levels of CI are sufficient for embryonic but not larval

**DISCUSSION**

Mutations in the cg gene result in an anterior overgrowth phenotype in leg imaginal discs that is similar to that produced by ubiquitous expression of hedgehog. The cg mutant phenotype is HH independent. Although this phenotype is very similar to other mutants such as ptc, which encode for negative regulators of the HH signaling pathway, it differs in one main respect: viable cg mutants reveal that CG also functions in the posterior compartment because adult wings have defects in venation here. Loss or gain of HH signaling has no effect in the posterior where the outputs are normally repressed by EN/INV.

**CG regulates ci expression**

An understanding of the cg mutant phenotype, why it causes overgrowth in the anterior and defects in differentiation in the posterior, comes not from the HH signaling pathway itself but from analysis of the protein primarily regulated by this pathway: CI. The primary function of HH signaling is to regulate the transcriptional activity of CI: to antagonize its degradation to a repressor and to maintain it as an activator (Aza-Blanc and Kornberg, 1999). A genetic interaction between cg and some ci mutants was identified many years ago (House, 1953, 1961; Waddington, 1953). The posterior wing

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**Fig. 6.** Phenotype of cgA22 and cg374 mutants. (A,B) Adult wings from transheterozygotes with en59. (A) + cg374/en59 flies have a fully penetrant loss of vein IV phenotype. (B) + cgA22/en59 flies only very rarely (>1%) show a gap in vein IV. (C-E) leg discs and (F) wing discs from cgA22 mutant larvae stained for al, dpp and ci expression. The phenotype of cgA22 is indistinguishable from cg07659.
development or that cg RNA is maternally supplied. The first possibility is supported by the observation that hypomorphic ci mutants (Fig. 4) are not embryonic lethal and survive to the early pupal stage. However, in situ analysis reveals that cg RNA is maternally supplied so that the question of whether cg is required during embryogenesis will require the generation of germline clones.

Full-length CI acts as a transcriptional activator and there is evidence that the lowered levels of CI in cg mutants also compromises CI function as an activator. Although, dpp is misexpressed in cg discs, the level of expression, even at the compartment border, is lower than that found in wild-type discs. A similar phenomenon has been demonstrated for loss of ci in the wing and it appears that the high levels of dpp in wild-type discs require activation by CI-155, as well as the absence of CI-75 (Method and Basler, 1999). Thus, the lower levels of dpp in cg discs are presumably due to lower levels of CI-155. Another gene directly activated by CI is en in late third instar wing discs. CI-dependent en activation in the anterior compartment (Blair, 1992; Strigini and Cohen, 1997) does not occur in cg mutant cells, again presumably because the level of the CI-155 activator form is too low.

The anterior/posterior (A/P) lineage restriction appears to be maintained by adhesive differences between A and P cells; this requires both CI in the anterior and EN in the posterior, and it has been proposed that CI activates and EN represses expression of an adhesion molecule that regulates cell mixing (Dahmann and Basler, 2000). In cg mutant discs, although CI levels across the compartment border are almost uniform, the lineage restriction is maintained, presumably because EN repression overcomes any activation of this adhesion molecule by the CI present in the posterior compartment.

Mechanism of CG regulation of ci

We have shown that CG is required to activate ci expression to its normal levels in the anterior compartment and to repress ci expression in the posterior. The CG protein contains multiple zinc fingers and is most probably a DNA-binding protein that would be expected to bind to elements at the ci locus. However, understanding the mechanism by which it regulates ci expression requires further studies. It is possible that CG functions as a standard transcription factor and activates ci transcription in the anterior and represses it in the posterior. If this is the case, its activity must be modified in either the anterior or posterior compartments. Analysis of the CG protein outside of the zinc fingers does not reveal any classical activator or repressor domains, but as these are often not well defined it is impossible to determine whether the protein has these activities without more-detailed studies.

An argument against such a direct involvement of CG in transcription is the well-documented role of EN in regulating ci expression. EN is a transcription factor that represses expression of several genes including ci, dpp and wg, and has been shown to bind to elements at the ci locus (Schwartz et al., 1995). It would appear likely that EN is the primary factor that represses transcription of ci in the posterior. If this is the case, the function of CG in regulating transcription may be indirect and may be to assist the binding of other transcription factors to the ci gene. If so, the misexpression of CI in the posterior of cg mutant discs would be due to a lowered ability of EN to bind in the absence of CG protein, while the lowered CI levels in the anterior would be due to a lowered ability of an, as yet unidentified, transcriptional activator of ci to bind. There are several possible mechanisms by which CG might affect the binding of other factors. For example, there may be direct physical interactions between CG and these other factors. Alternatively, CG action could be more indirect, for example, it could modify chromatin structure at the ci locus producing a more open conformation. Further studies are required to test these possibilities.

Given the similarity between HH signaling and regulation of CI/Gli protein activity in Drosophila and vertebrates (Murone et al., 1999; Ruiz i Altava, 1999), it will be important to determine whether the transcriptional activity of the Gli genes is also regulated by a CG homolog. Disruption of HH signaling or activity of Gli proteins can result in different human pathologies that include specific types of cancer or limb abnormalities (Ingham, 1998; Ruiz i Altava, 1999), indicating that individuals with mutations in a human cg homolog may exhibit similar diseases.

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REFERENCES


Dahmann, C. and Basler, K. (2000). Opposing transcriptional outputs of...
Hedgehog signaling and engrailed control compartmental cell sorting at the Drosophila A/P boundary. *Cell* **100**, 411-422.


