EGFR signalling inhibits Capicua-dependent repression during specification of *Drosophila* wing veins

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**SUMMARY**

Localised activation of the Ras/Raf pathway by Epidermal Growth Factor Receptor (EGFR) signalling specifies the formation of veins in the *Drosophila* wing. However, little is known about how the EGFR signal regulates transcriptional responses during the vein/intervein cell fate decision. We provide evidence that EGFR signalling induces expression of vein-specific genes by inhibiting the Capicua (Cic) HMG-box repressor, a known regulator of embryonic body patterning. Lack of Cic function causes ectopic expression of EGFR targets such as *argos*, *ventral veinless* and *decapentaplegic* and leads to formation of extra vein tissue. In vein cells, EGFR signalling downregulates Cic protein levels in the nucleus and relieves repression of vein-specific genes, whereas intervein cells maintain high levels of Cic throughout larval and pupal development, repressing the expression of vein-specific genes and allowing intervein differentiation. However, regulation of some EGFR targets such as *rhomboid* appears not to be under direct control of Cic, suggesting that EGFR signalling branches out in the nucleus and controls different targets via distinct mediator factors. Our results support the idea that localised inactivation of transcriptional repressors such as Cic is a rather general mechanism for regulation of target gene expression by the Ras/Raf pathway.

Key words: Capicua, Bullwinkle, Ras/Raf pathway, Transcriptional repression, Vein development, *Drosophila*

**INTRODUCTION**

Activation of receptor tyrosine kinases (RTKs) by secreted growth factors regulates cell proliferation and cell fate determination in many different cellular and developmental contexts. Despite such widespread functions, RTK signalling involves a common cytoplasmic cascade including Ras, and the serine-threonine kinases Raf, MAPKK/MEK and MAPK. Thus, a central issue is how activation of the same cytoplasmic components elicits different responses depending on the cell context (Hill and Treisman, 1995; Simon, 2000). The nuclear response to the signal depends on many cases on the availability of direct transcription factors for direct phosphorylation by the Ras/Raf pathway. In addition, RTK signalling can trigger mechanisms of either activation or repression of gene expression, adding an extra level of complexity to the final output. Thus, cell responses to RTK signalling are largely determined by the mechanisms and factors that mediate transcriptional regulation downstream of the RTK cascade.

A well-established model of RTK signalling concerns the formation of veins in the *Drosophila* wing. The insect wings consist of a bilayer of epidermal cells closely apposed by their basal sides that secrete a thin and transparent cuticle. In *Drosophila*, wings display a stereotyped pattern of five longitudinal veins and two transverse crossveins. The veins are specialised epithelial cells present in both wing surfaces. During late differentiation, dorsal and ventral veins match accurately providing a hollow space for the passage of axons and trachea (García-Bellido and de Celis, 1992).

Vein tissue specification occurs during larval and pupal stages within the wing imaginal discs, and involves a complex network of gene regulation. Central to this network is the Epidermal Growth Factor Receptor (EGFR) RTK (Díaz-Benjumea and Hafen, 1994), which, in *Drosophila*, functions in a wide spectrum of developmental processes such as oogenesis, patterning of embryonic structures such as the ventral epidermis and trachea, and specification of photoreceptor cells in the compound eye (Schweitzer and Shilo, 1997). In wing discs, activation of EGFR in rows of cells causes their determination as prospective wing veins. Consistent with this function, loss-of-function mutations in components of the EGFR pathway cause loss of veins, whereas gain-of-function alleles and/or overexpression of those components result in ectopic vein development (Díaz-Benjumea and Hafen, 1994).

The role of EGFR signalling in vein patterning involves complex molecular interactions that (1) restrict its activity to appropriate cells, and (2) impose cell fate changes in response to signalling. Activation of EGFR in precise rows of cells
depends on several signalling pathways that pattern the wing disc (de Celis and Barrio, 2000), and relies ultimately on the localised activity in the veins of different EGFR ligands and the Rhomboid (Rho) transmembrane protein, a mediator of EGFR activation (Schnepf et al., 1996; Sturtevant et al., 1993). Regulated EGFR activation specifies vein fates by both activation and repression of several downstream target genes. These encode transcription factors and also signalling molecules, such as the Decapentaplegic (Dpp) TGFβ fly homologue, that have to be activated in the veins for their proper differentiation (de Celis, 1997).

Although a broad picture of how veins are specified is emerging, little is known about how the EGFR cascade regulates target gene expression in the nucleus of developing vein cells. In this work, we present evidence that the EGFR pathway functions, at least in part, by promoting destabilisation of the Capicua (Cic) protein in vein nuclei. Cic is an HMG-box transcription factor that controls early embryonic patterning in response to Torso RTK signalling. Cic functions as a repressor of two genes expressed in the terminal regions of the embryo, tailless (tl) and huckebein (hkb). Cic accumulates in central regions of the embryo, but is under negative post-transcriptional control by Torso signalling at the termini, thus permitting expression of tl and hkb at the embryonic poles (Jiménez et al., 2000).

Our results support a similar regulation of the Cic protein by EGFR signalling in the wing. We have found that Cic activity prevents differentiation of vein tissue in the wing pouch. In turn, restricted activation of the Ras/Raf pathway in the presumptive veins antagonises Cic repressor activity in this tissue, thus allowing localised expression of genes mediating vein differentiation. We discuss the role of Cic as a general transcriptional repressor regulated by the Ras/Raf pathway.

MATERIALS AND METHODS

Fly stocks

We have used the following genetic variants: the cic<sup>2</sup> allele (formerly E(Dl)D49) (Klein and Campos-Ortega, 1992), cic<sup>1</sup>, bwk<sup>β8</sup> and bwk<sup>β11</sup> (Jiménez et al., 2000), bwk<sup>β82</sup>, bwk<sup>β14</sup> and bwk<sup>β11</sup> mutants (Rittenhouse and Berg, 1995). We also used the second chromosome insertion I<sup>wt</sup>: cic::fla<sup>60</sup>, a fla-tagged version of the rescuing construct containing the cic complete coding sequence (Jiménez et al., 2000) (see Fig. 1). As loss of function alleles in the Ras/Raf pathway, we used the Ras<sup>1</sup> null allele Ras<sup>Ac26b</sup> (Hou et al., 1995). EGFR ectopic activation was achieved by overexpressing the protein Vin (Vn) with an UAS Vn (Schnepf et al., 1996) and the EngrailedGAL4<sup>21A</sup> line (Brand, 1997). Other mutants used were the rho<sup>10</sup> and vin<sup>11</sup> combination (Díaz-Benjumea and García-Bellido, 1990) and the argos<sup>11</sup> lacZ reporter (Freeman et al., 1992). To counterstain the cell nuclei in some preparations, staining was carried out in flies expressing ubiquitous nuclear GFP (Davis et al., 1995).

Characterisation of the cic<sup>2</sup> allele

The lethal mutation cic<sup>2</sup> was crossed with several deficiencies of the third chromosome deficiency kit (kindly provided by C. Balayo), covering the 92B3-95D6 interval. Of these, Df(3R)Dl-BX12 and Df(3R)Hb-79 were found to uncover cic<sup>2</sup>, mapping the mutation to region 92B-D. Further crosses with cic and bwk alleles confirmed that cic<sup>2</sup> belongs to these related complementation groups. To characterise cic<sup>2</sup> molecularly, genomic fragments corresponding to the mutant gene were amplified by PCR and sequenced directly.

Clonal analysis

Adult cic mutant FRT/FLP (Xu and Rubin, 1993) M<sup>+</sup> clones marked with the forked mutation were induced in males of the following genotypes: yhsFLP<sup>65a</sup>: FRT82B UbgGFP83 <sup>0<sup>67D</sup> M(3)95A/FRT82B bwk<sup>β11</sup> e<sup>1</sup> and yhsFLP<sup>65a</sup>: FRT82B UbgGFP83 <sup>0<sup>67D</sup> M(3)95A/FRT82B cic<sup>2</sup> e. Clones mutant for Ras-1 and the double mutant clones Ras-1/cic were induced, respectively, in males of the genotype yhsFLP<sup>65a</sup>: FRT82B UbgGFP83 <sup>0<sup>67D</sup> M(3)95A/FRT82B Ras<sup>Ac26b</sup> and yhsFLP<sup>65a</sup>: FRT82B UbgGFP83 <sup>0<sup>67D</sup> M(3)95A/FRT82B Ras<sup>Ac26b</sup> cic<sup>2</sup> e. The expression of the argos lacZ reporter was monitored in cic mutant clones generated in flies with the genotype yhsFLP<sup>65a</sup>: FRT82B UbgGFP83 <sup>0<sup>67D</sup> M(3)95A/FRT82B cic<sup>2</sup> e. Clones were generated at 60±12 hours AEL with a 5 minutes heat shock pulse at 37°C.

Staining procedures

Immunostaining was performed according to standard protocols. For staining of Cic protein we used a polyclonal antibody from rat (1/300). Other primary antibodies were: mouse anti-Bs (1/600) (kindly provided by E. Martin-Blanco), rat anti-Vvl (1/300) (Llimargas and Casanova, 1997), mouse anti-β-gal (1/100) (Promega) and rabbit anti-β-gal (1/1000) (Promega). Secondary antibodies include anti-rat and anti-mouse conjugated to fluorescein and Texas Red and anti-rabbit conjugated to Cy5, all of them diluted 1/200 (Jackson Laboratories). Fluorescence was visualised in a Leica TCS confocal microscope. In situ hybridisation was carried out according to Sturtevant et al. (Sturtevant et al., 1993) with DIG-labelled antisense riboprobes made with the full CDNAs of the dpp, rho and cic genes.

RESULTS

capicua and bullwinke mutations affect a single gene

The E(Dl)D49 mutation was identified in a screen for dominant enhancers of the wing vein phenotype of Dl/+ flies (Klein and Campos-Ortega, 1992). E(Dl)D49 is a recessive lethal mutation, but the heterozygous flies present with variable penetrance small patches of extravein tissue in the distal part of the L5 wing vein. Using standard recombination and deficiency tests, E(Dl)D49 was mapped to the 92B-D cytological region, in the right arm of the third autosome (see Materials and Methods). Crosses with known mutations in the same region showed that E(Dl)D49 is allelic to two sets of mutations: bullwinke (bwk) and capicua (cic) (Rittenhouse and Berg, 1995; Jiménez et al., 2000). For convenience, we renamed the E(Dl)D49 allele cic<sup>2</sup>.

The original bwk allele, bwk<sup>β82</sup>, is a P-element-induced female sterile mutation that affects the patterning of the ovary follicle cells and the early embryo, giving rise to embryos with abnormal eggshells that also show a bicaudal phenotype (Rittenhouse and Berg, 1995). bwk<sup>β82</sup> also has zygotic effects: homozygous adult flies display ectopic vein tissue in their wings (Rittenhouse and Berg, 1995). In contrast, the original cic<sup>1</sup> allele is a female sterile mutation caused by the insertion of a hobo transposon 300 bp away from the bwk<sup>β82</sup> insertion (Fig. 1). Homozygous cic<sup>1</sup> females produce embryos with strongly suppressed trunk regions that are replaced by terminal structures (Jiménez et al., 2000).

The genetic relationship between cic and bwk mutations is unclear. Although cic<sup>1</sup> and bwk<sup>β82</sup> complement each other, both mutations are uncovered by specific small deficiencies (less than 500 bp) in the region (Jiménez et al., 2000). To help
**Fig. 1.** Diagram of the *cic* locus and several mutations affecting the gene. The organisation of the major maternal transcript is shown; coding and untranslated regions are depicted by black and white boxes, respectively. The *P(PZ)bwk8482* (P-element) and *cic* (hobo) transposon insertions are indicated by triangles. The *cic* allele maps ~380 bp upstream of the HMG-box region (hatched) and consists of a deletion of 13 nucleotides, tcgtatatgc. Fragments deleted by three independent *P(PZ)bwk8482*-derived excisions are indicated by bars (open ends denote uncertainties in their limits). The genomic region included in the *cic*+ rescuing construct is also indicated.

To clarify this issue, we analysed the phenotypes observed in heteroallelic combinations of several *cic* - and *bwk*-related alleles. This analysis shows that both *cic* and *bwk8482* mutations are not complemented by *cic* and specific P-element excision alleles obtained by mobilisation of *bwk8482* (e.g. *bwk81* and *bwk814*) (Fig. 1), resulting in *cic* and *bwk*-associated phenotypes, respectively (Table 1). Thus, *cic* and *bwk8482* behave as strong hypomorphs or nulls for both *cic* and *bwk* genetic functions. These observations, together with the proximity of the *bwk8482* and *cic* transposon insertions, raise the possibility that *cic* and *bwk* mutations disrupt different functions of a single gene. Alternatively, *bwk* and *cic* may be adjacent genes that are simultaneously affected by *cic* and some *bwk8482* derived excisions.

As *cic* is an EMS-induced mutation, we looked for a point molecular lesion in the *cic*-coding region associated to the *cic* chromosome, and identified a deletion of 13 bp causing a frameshift upstream of the Cic HMG box (Fig. 1). Thus, a complete absence of *cic* activity recapitulates all known defects of *cic* and *bwk* mutations, strongly indicating that all these mutations affect the *cic* gene. In this context, the *cic* and *bwk8482* mutations probably represent regulatory alleles that affect only specific *cic* functions. As the hobo element that causes the *cic* mutation is inserted within the first exon of the gene, we suspect that *bwk* specific functions depend on use of an alternative promoter located upstream of the *bwk8482* insertion (Fig. 1). Consistent with these ideas, a genomic construct containing the complete *cic* transcription unit and 1.2 kb of upstream region rescues the *cic* related terminal defects, and significantly reduces the extravein phenotypes of *cic*/*bwk81*, *cic*/*bwk8482* and *cic*/*bwk814* flies. However, this genomic construct fails to rescue the chorion phenotype of embryos laid by *bwk8482* females (Jiménez et al., 2000), possibly because it lacks specific regulatory regions required for *bwk*-associated functions (Rittenhouse and Berg, 1995).

**Cic is required during wing development for intervein specification**

To study the role of *cic* in wing development, we examined the effects of a complete loss of function of the gene in an heteroallelic combination of the *cic* allele with the deficiency *bwk811*, which removes the entire *cic*-coding region (Fig. 1). This combination is generally lethal, but adult scapers can be obtained with a low frequency. The most striking feature of these flies is their abnormal wings, which are reduced in size and often show blisters that separate the ventral and dorsal surfaces. The mutant wings display abundant extravein tissue, preferentially close to the normal veins, which appear thickened (Fig. 2A-D). This phenotype is a consequence of intervein cells acquiring morphogenetic features of vein cells, which are typically smaller, more pigmented and with shorter and thicker trichomes than wild-type intervein cells. However, close examination reveals that the transformation to vein tissue is not complete and many intervein cells acquire intermediate morphologies between those shown by typical vein and intervein cells (Fig. 2C,D). In addition, some regions such as the area between veins L3 and L4 never differentiate as vein tissue in mutant wings, indicating that specification of intervein fate in this territory is independent of *cic* (see Discussion).

Wild type, adult flies of the corresponding genotype are morphologically normal and fertile; terminal, adult females morphologically normal but laying embryos with transformations from trunk to terminal parts; extra vein, adult wings differentiating ectopic veins; *bwk*, adult females lay few eggs displaying eggshell defects; almost lethal, cases where the female progeny could not be scored.

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<th>Table 1. Phenotypes of mutant alleles</th>
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<td>Mutant allele</td>
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<td><em>cic</em>&lt;sup&gt;1&lt;/sup&gt;</td>
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<td><em>bwk</em>&lt;sup&gt;8482&lt;/sup&gt;</td>
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<td><em>bwk</em>&lt;sup&gt;814&lt;/sup&gt;</td>
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<sup>*</sup>Although the original *bwk*<sup>8482</sup> mutation is homozygous viable (Rittenhouse and Berg, 1995), in our hands, the *bwk*<sup>8482</sup>-bearing chromosome now behaves as a homozygous lethal.

<sup>+</sup>Wild type, adult flies of the corresponding genotype are morphologically normal and fertile; terminal, adult females morphologically normal but laying embryos with transformations from trunk to terminal parts; extra vein, adult wings differentiating ectopic veins; *bwk*, adult females lay few eggs displaying eggshell defects; almost lethal, cases where the female progeny could not be scored.

<sup>1</sup>*cic*, mutation that affects only the terminal function; *bwk*<sup>8482</sup>, mutations that affect the wing and Bwk functions, but not the terminal; *bwk*<sup>814</sup>, *bwk*<sup>811</sup>, *cic*<sup>2</sup>, *bwk*<sup>811</sup>, mutations that affect all three functions.
opposite direction to those of wild-type males, as if the whole structure was rotated 180° (not shown). This phenotype could result from defects in the musculature associated to the male genitalia, but we have not studied it in detail.

To analyse the effects of cic in vein development further, we generated clones homozygous for either cic^2 or bwk^∆11 mutations with the Minute technique, which allows the generation of clones that cover large territories (Morata and Ripoll, 1975) (see Materials and Methods). Both sets of experiments gave similar results; we describe below those corresponding to cic^2. In our analysis, we studied 45 clones, covering the complete wing surface. The mutant clones are consistently smaller than their respective controls, suggesting that proliferation of cic^2 mutant cells is impaired. Mutant cells often differentiate as vein tissue in ectopic positions, particularly if they are close to normal veins (Fig. 2E-G). However, these changes in cell fate are not strictly cell autonomous, as many intervein cells within the mutant clones differentiate normally. This could be due in part to a rescue of the phenotype by wild-type cells situated in the other surface of the wing, because mutant clones that occupy only one wing surface display always less extravein tissue than clones occupying both dorsal and ventral surfaces (Fig. 2G). In addition, mutant clones can also induce neighbouring wild-type cells to differentiate as vein tissue, either in the same wing surface or in the opposite one (Fig. 2F,H). We conclude that cic functions as a negative regulator of vein differentiation in intervein territories, probably as part of a network involving cell interactions.

Post-transcriptional regulation of Cic protein in the wing disc

We next examined the expression pattern of cic in wild-type wing discs. In situ hybridisation of third instar discs showed uniform distribution of cic transcripts (Fig. 3A). By contrast, staining of similar discs with a specific Cic antibody revealed a complex pattern of protein accumulation: Cic accumulates at high levels in the wing pouch and in the primordial hinge region, but not in the notum region (Fig. 3B). At this stage, Cic levels begin to drop in the presumptive third longitudinal vein and in two rows of cells running along the D/V boundary, which correspond to the future wing margin (Fig. 3B,C). Moreover, the remaining Cic protein in those cells is cytoplasmic, whereas in other regions of the wing pouch (and in the adjacent peripodial cells), Cic is clearly nuclear (Fig. 3C,D). During pupariation [from 6 to 34 hours after puparium formation (APF)], Cic levels also decline in all presumptive longitudinal wing veins and crossveins (Fig. 3E-G). This specific accumulation of Cic in intervein sectors is consistent with its role as a negative regulator of vein differentiation.

The Cic protein is downregulated by the Ras/Raf pathway in the wing disc

In the early embryo, Cic is under negative post-transcriptional regulation by the Ras/Raf signalling pathway (see Introduction). Several lines of evidence are consistent with a similar mechanism of Cic inactivation in the wing. First, Ras/Raf signalling in response to EGFR activation is required for vein differentiation (Díaz-Benjumea and Hafen, 1994), and levels of
EGFR signalling inhibits Cic-dependent repression

997

Active, diphosphorylated MAPK are maximal in presumptive veins (Gabay et al., 1997), where Cic protein is downregulated. Furthermore, both ectopic activation of the Ras/Raf pathway and loss of cic function cause similar extravein phenotypes (Brunner et al., 1994). Thus, it is conceivable that Ras/Raf signalling induces vein differentiation by downregulating Cic protein in prospective vein cells.

If this hypothesis is correct, Ras/Raf signalling should be dispensable, at least in part, for vein differentiation in the absence of cic activity. We tested this idea in two different ways. In one case, we eliminated Ras/Raf signalling in the wing by mutating factors required for EGFR activation. The hypomorphic viable combination rho ve vn 1 affects the activity in the wing of both the vein (vn) locus, which encodes a putative EGFR ligand (Schnepp et al., 1996), and rho, which encodes a transmembrane protein involved in EGFR activation (Sturtevant et al., 1993). In flies mutant for this combination, the activation of MAPK in the presumptive vein cells is prevented (Martín-Blanco et al., 1999) and as a consequence, veins fail to differentiate (Fig. 4A). By contrast, flies with the triple mutant combination rho ve vn 1 cic 2/rho ve vn 1 bwk Δ11 display the same extravein phenotypes as cic 2/bwk Δ11 mutant wings (Fig. 4B,C), suggesting that Cic functions downstream of EGFR activation. We generated large M+ clones homozygous for the RasΔC100B and cic 2 null alleles. Mutant clones lacking only the Ras-1 (Ras85D–FlyBase) function show the expected loss of vein tissue (Fig. 4D), but this effect is suppressed in clones lacking both Ras-1 and cic activities, which show ectopic vein development (Fig. 4E,F). These results support the notion that Ras/Raf signalling inhibits cic function during vein differentiation.

To test directly if activity of the Ras/Raf pathway mediates downregulation of Cic protein in veins, we monitored its distribution in clones homozygous for RasΔC100B. Mutant clones induced in second larval instar were examined in early pupal discs, where Cic protein levels begin to decline in all the differentiating veins. In 0-6 hours APF mosaic wings, Cic is autonomously expressed at high levels within the Ras mutant clones, even when they occupy positions corresponding to the normal veins (Fig. 4G,H). Furthermore, Cic levels are higher in the mutant clones than in the rest of the wing, suggesting that Ras activity also exerts basal downregulation on Cic in intervein cells. We also performed the converse experiment and tested the effect of the hyperactivation of EGFR signalling on Cic accumulation. We used the GAL4/UAS system to overexpress the EGFR activator Vein in the posterior compartment of pupal wings (Materials and Methods). As shown in Fig. 4I, this caused a significant reduction of Cic protein levels in the posterior compartment of mutant wings by 0-6 hours APF. Altogether, these genetic and
immunohistochemical data indicate that the Cic protein is negatively regulated by EGFR signalling during wing vein specification.

**Cic acts as a repressor of wing vein-specific genes**

The above results suggest that EGFR signalling activates the expression of genes involved in vein patterning by antagonising *cic* repressor activity in presumptive vein cells. To test this hypothesis, we examined the role of *cic* in regulating expression of genes induced by the EGFR pathway. One such gene is *aos*, which encodes a secreted factor that mediates
negative feedback modulation of EGFR signalling in many developmental processes (Golembio et al., 1996; Wasserman and Freeman, 1998). In the wing, aos is expressed specifically in presumptive vein cells, where EGFR activity is maximal (Gabay et al., 1997) (Fig. 5A). We analysed expression of an aos-lacZ reporter gene in third larval instar and pupal cic^2/bwk^A11 mutant discs and found a dramatic derepression compared with the wild-type pattern. Ectopic aos-lacZ expression is observed throughout the wing pouch, except in the presumptive wing margin (Fig. 5B). This ectopic expression is maintained in intervein cells until at least 30 hours APF (Fig. 5D). However, we note that in cic mutant discs, aos is expressed at higher levels in presumptive vein cells than in intervein territories, suggesting that cic is not the only factor involved in aos regulation (Fig. 5B). We have also analysed whether aos expression is repressed autonomously by cic. We find that in clones homozygous for the cic^2 mutation, the aos-lacZ reporter is expressed by all the cells within the clones (Fig. 5E,F). Thus, cic behaves in a cell autonomous way as a repressor of aos transcription in intervein cells.

Cic could also regulate the expression of vvl, which encodes a POU domain transcription factor required for vein differentiation (de Celis et al., 1995). Double mutants for EGFR and vvl display synergistic phenotypes (de Celis et al., 1995), indicating that these two elements interact genetically. Indeed, vvl expression in the wing is regulated by Ras/Raf signalling (J. F. de Celis, personal communication). In wild-type wing discs, Vvl protein is distributed rather uniformly during larval stages, but it becomes restricted to vein cells during early pupal development (Fig. 6A), so that by 24-30 hours APF, the protein is no longer detectable in intervein cells (Fig. 6C,E). By contrast, Vvl protein persists in intervein cells of cic^2/bwk^A11 mutant discs from 6 to 30 hours APF, albeit at variable levels (Fig. 6B,D,F). Thus, cic activity is necessary for repression of vvl in intervein regions of early pupal discs.

Another gene activated in the veins by the Ras/Raf signalling is rho (Sturtevant et al., 1993; Martín-Blanco et al., 1999). In the wing, rho activity participates in a positive feedback loop that boosts EGFR activation in vein cells (Martín-Blanco et al., 1999). We examined rho expression in cic^2/bwk^A11 wing discs at different stages of development. A normal rho pattern was observed during late larval and early pupal stages (not shown), but clear ectopic expression was detected in intervein regions by 24-30 hours APF. This ectopic expression precedes the differentiation of extravens in the adult mutant wings (Fig. 6G,H). Thus, cic is required, either directly or indirectly, for rho repression in intervein cells during late pupal stages.

The differentiation of vein cells during pupal stages also requires the function of the secreted Dpp factor, that is implicated in the refinement of vein patterning during pupal development (de Celis, 1997). In wild-type pupal wings, dpp transcripts accumulate in precise stripes corresponding to the developing veins (Fig. 6I). By contrast, such transcripts are clearly observed throughout the wings of cic^2/bwk^A11 mutants by 24-30 hours APF (Fig. 6J). Dpp levels are still higher in regions that will give rise to vein tissue in the mutant, but there is ectopic dpp expression in most of the future intervein cells, indicating that cic affects dpp expression in late pupal wings. This effect on a secreted factor that diffuses from its source may explain, at least in part, the non autonomous phenotypes of cic mutant clones (see Discussion).

The above results indicate that Cic is required during larval and pupal stages for repression in intervein cells of several vein-specific genes. We also checked if the expression of intervein genes is affected in the absence of Cic. One such gene is blistered (bs), which encodes a homologue of the
mammalian SRF transcription factor (Montagne et al., 1996) and is autonomously required for intervein differentiation (Fristrom et al., 1994). In wild-type discs, Bs and the Cic repressor are co-expressed in all intervein cells throughout late larval and early pupal stages (not shown). We find that Bs expression is normal in third larval instar and 6 hours APF pupal discs of cic2/bwkM11 mutant flies (not shown), indicating that cic is not required for bs regulation at these stages. However, Bs levels are clearly reduced in intervein cells at 24-30 hours APF (Fig. 6D). As Cic appears to be a dedicated repressor, it seems unlikely that it could contribute directly to the maintenance of Bs expression in the same cells; rather, we interpret this late repression of bs as an indirect effect caused by ectopic expression of vein-specific genes in intervein territories of these mutant discs. Indeed, it has been shown that ectopic expression of rho in intervein cells causes downregulation of bs during pupal stages (Roch et al., 1998).

The analysis of Bs and Vvl expression led also to the interesting observation that both proteins are co-expressed in many cells throughout the wing pouch of cic2/bwkM11 mutants (Fig. 6D,F), whereas they show almost perfect complementary expression patterns in wild-type discs (Fig. 6C,E). The co-expression of vein and intervein markers, such as Dpp, Vvl and Bs could explain the combination of vein and intervein cell morphologies observed in cic2/bwkM11 adult wings.

DISCUSSION

The Cic HMG-box repressor plays a key role in regulation of embryonic terminal patterning under the control of Torso RTK signalling (Jiménez et al., 2000). We have shown that Cic participates also in other developmental processes, such as specification of vein cell fates in the wing. Our genetic data indicate that cic terminal-class alleles and the bwk group of mutations are hypomorphic alleles disrupting specific aspects of cic function, whereas the null allele cic2 and several available deletions affect all functions of the gene. We provide evidence that Cic mediates wing vein patterning acting as a repressor of vein-specific genes downstream of the EGFR signalling pathway.

Role of Cic in wing vein development

There are two key aspects of Cic function as a developmental regulator: its ability to repress specific target genes in defined territories, and its inhibition by the Ras/Raf pathway to allow expression of those targets in complementary positions. In the blastoderm embryo, Cic is required for development of trunk body regions and represses genes mediating differentiation of terminal structures. Torso RTK activation at each pole of the embryo alleviates Cic-dependent repression and initiates the terminal gene expression program (Jiménez et al., 2000). Our results support a similar model for cic function during specification of vein versus intervein fate in the wing. Loss of cic function in the wing causes formation of ectopic vein tissue, implying that Cic mediates intervein specification by restricting vein formation to appropriate regions. In intervein territories, Cic behaves as a repressor of vein-specific genes such as aos and vvl but does not seem to affect directly the expression of bs, which is required for the specification of intervein fates. Finally, EGFR signalling leads to downregulation of Cic protein levels in vein nuclei, thus relieving Cic-mediated repression and promoting vein development (Fig. 7).

Nevertheless, several data suggest a more complex regulation of vein specification compared to terminal patterning. First, we have shown that expression of rho, a positive target of EGFR signalling in the wing and other tissues (Martín-Blanco et al., 1999; Hsu et al., 1996), is not affected by cic during third larval instar and early pupariation (Fig. 7). This suggests that EGFR signalling can mediate activation of some targets in the wing disc by mechanisms other than Cic inhibition. Similarly, the EGFR pathway has been shown to repress bs expression in presumptive vein cells (Roch et al., 1998), a process that is independent of Cic (Fig. 7). These results imply that different transcription factors act downstream of the EGFR cascade to direct changes in gene expression during patterning of wing veins. Indeed, recent results indicate that EGFR signalling activates certain target genes via direct phosphorylation of Fos protein (Ciapponi et al., 2001).

Moreover, vein differentiation is not a mere result of EGFR activation but depends on other signals such as Dpp and Notch (de Celis, 1997; de Celis et al., 1997), and on the distribution of additional transcription factors that contribute to wing patterning. For example, the Collier/Knot nuclear factor has been shown to induce high levels of Bs expression between veins L3 and L4, promoting intervein development in this region (Vervoort et al., 1999). All these inputs are linked in a complex circuit of intercellular signalling and gene regulation that progressively refines vein determination during late larval and pupal development. This signalling network could provide an explanation for the observed non-autonomy of cic phenotypes.
during vein specification. Thus, although cic represses aos expression in a cell-autonomous manner, this and other cic targets are likely to participate in signalling mechanisms that affect adjacent cells. Consistent with this idea, we find that cic mutant cells express ectopic Dpp product, a diffusible factor that promotes vein differentiation (de Celis, 1997).

We have shown that in cic mutant wings, many cells differentiate, acquiring morphological features that are intermediate between those observed in either vein and intervein cells. In these wings, most cells co-express Bs and Vvl proteins, which are normally restricted to vein and intervein cells, respectively, suggesting that vein/intervein fate specification may result from a balance of these factors rather than on a simple binary switch. In this context, the concerted activities of signalling cascades such as Dpp, Notch and Ras/Raf pathways may regulate cell differentiation by modulating the balance of nuclear factors that act in a dose-dependent way. This hypothesis provides a mechanism that could explain the enormous variability observed in the cell morphologies of different insect wings.

**Role of Cic as a general repressor downstream of RTK signalling**

In this work, we have shown that Cic acts in wing development in a similar way to that previously described in the early embryo. Moreover, the fact that mutant clones for the Groucho repressor display extraveneis, similarly to cic clones (de Celis and Ruiz-Gómez, 1995), indicates that these two proteins could interact as partners during wing development, as it is the case during embryonic development (Jiménez et al., 2000). Indeed, we have observed weak genetic interactions between different cic and gro alleles during wing development (F. R., unpublished). Thus, Cic and Gro could be part of a conserved repressor complex downregulated by the Ras/Raf molecular cassette in different cellular contexts. In this regard, the phenotype of bwk mutations suggests that Cic may also function as a target of other RTK signals during patterning of the eggshell in the ovary (Rittenhouse and Berg, 1995). However, it should be noted that cic does not seem to act in all developmental processes mediated by Ras/Raf signalling in *Drosophila*. For example, the eyes of cic mutant flies appear normal (F. R., unpublished), even though the Ras/Raf pathway controls several aspects of cell fate specification and patterning in this tissue (Freeman, 1997). These observations support the idea that the Ras/Raf pathway can regulate cell specification in a cic-independent way depending on the cell context.

Previous work has shown that during patterning of ovary follicle cells, the expression of rho is controlled by the Ras/Raf pathway via another transcriptional repressor, the CF2 protein (Hsu et al., 1996). CF2 is tagged for cytoplasmic retention and degradation after direct phosphorylation by MAPK (Man trova and Hsu, 1998). The Cic protein has also consensus sites for phosphorylation by MAPK (Jiménez et al., 2000), suggesting that Cic levels could be regulated post-transcriptionally in a similar way to CF2. This indicates that localised downregulation of specific repressors is a common mechanism for the activation of target genes by the Ras/Raf pathway. The identification of highly conserved cic homologues in mice and humans suggests that regulation of gene expression by RTK signalling in vertebrates may also involve relief of Cic-dependent repression.

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**REFERENCES**


