A dual role for homothorax in inhibiting wing blade development and specifying proximal wing identities in Drosophila

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SUMMARY

The Drosophila wing imaginal disc gives rise to three body parts along the proximo-distal (P-D) axis: the wing blade, the wing hinge and the mesonotum. Development of the wing blade initiates along part of the dorsal/ventral (D/V) compartment boundary and requires input from both the Notch and wingless (wg) signal transduction pathways. In the wing blade, wg activates the gene vestigial (vg), which is required for the wing blade to grow. wg is also required for hinge development, but wg does not activate vg in the hinge, raising the question of what target genes are activated by wg to generate hinge structures. Here we show that wg activates the gene homothorax (hth) in the hinge and that hth is necessary for hinge development. Further, we demonstrate that hth also limits where along the D/V compartment boundary wing blade development can initiate, thus helping to define the size and position of the wing blade within the disc epithelium. We also show that the gene teashirt (tsh), which is coexpressed with hth throughout most of wing disc development, collaborates with hth to repress vg and block wing blade development. Our results suggest that tsh and hth block wing blade development by repressing some of the activities of the Notch pathway at the D/V compartment boundary.

Key words: Proximal-distal axis, Wing development, Wing hinge, homothorax, wingless, Notch, vestigial, Drosophila, extradenticle

INTRODUCTION

The wing imaginal disc of Drosophila gives rise to the mesonotum, the major portion of the fly notum, and its associated appendage, the wing (Bryant, 1978). The wing is composed of a more distal portion, called the wing blade, and a more proximal region, called the wing hinge (Fig. 1A). The mesonotum, wing hinge and wing blade are derived from different positions along the proximo-distal (P-D) axis of the wing disc (Fig. 1B). Although much is known about the control of wing blade development (see below), relatively little is known about the formation of the wing hinge, or how cells within the wing imaginal disc are allocated to form either the mesonotum, wing hinge or wing blade. To provide answers to these questions, the mechanisms that govern positional information along the P-D axis in the wing must be investigated.

Wing development requires wingless (wg), which encodes a member of the Wnt family of secreted proteins (reviewed in Klingensmith and Nusse, 1994). In the absence of wg function, neither the wing blade nor hinge are formed, and a duplication of the notum results (Sharma and Chopra, 1976; Morata and Lawrence, 1977). wg is first expressed in the wing imaginal disc during second instar in a small ventral-anterior patch of cells (Couso et al., 1993; Williams et al., 1993; Ng et al., 1996). At about this same time in wing disc development, a cell lineage restriction arises in wing discs that separates the dorsal compartment (D) from the ventral compartment (V) (Garcia-Bellido et al., 1973). This lineage restriction is due to the expression of the LIM homeobox gene aperous in the dorsal compartment (Diaz-Benjumea and Cohen, 1993; Blair et al., 1994). The D/V boundary divides the wing blade, hinge and notum regions of the wing disc (Fig. 1B).

The D/V boundary plays an important role in wing development by providing a source of both short- and long-range signals. The Notch signaling pathway is activated on both sides of the D/V boundary and, in the wing blade, activates expression of wg at the D/V boundary (Diaz-Benjumea and Cohen, 1995; Kim et al., 1996; Neumann and Cohen, 1996b; Klein and Martinez-Arias, 1998). Both Notch and wg activate the gene vestigial (vg) which, together with its cofactor encoded by scalloped (sd), is essential for the growth of the wing blade (Williams et al., 1991, 1994; Kim et al., 1996; Neumann and Cohen, 1997; Halder et al., 1998; Simmonds et al., 1998; Varadarajan and VijayRaghavan, 1999). Importantly, the ways in which Notch and wg activate vg are very different. Notch activates vg in cells very close to the D/V boundary, both within and outside the wing blade, by activating an enhancer called the vg-boundary enhancer (vgBE) (Williams et al., 1994; Kim et al., 1996) (Fig. 1B). In contrast, wg, secreted from cells at the D/V boundary, induces vg expression at a distance from the wg-secreting cells, thereby providing positional information along the P-D axis within the wing blade (Zecca et al., 1996; Neumann and Cohen, 1997). The result is a...
gradient of vg expression inside the wing blade, with peak levels at the D/V boundary. In the wing blade, cells at the D/V boundary give rise to the wing margin, and both Notch and wg signaling at the D/V boundary are important for the generation of this specialized structure and its associated sensory organs (Cousou et al., 1994; Williams et al., 1994; Diaz-Benjumea and Cohen, 1995; Rulifson and Blair, 1995; Neumann and Cohen, 1996b).

The gene regulatory rules outside the wing blade are different from those inside the wing blade. For example, outside the wing blade in the posterior compartment, cells along the D/V boundary express vg, but not wg (Fig. 1B). Instead of being activated at the D/V boundary, wg is expressed in two concentric rings that surround the wing blade; these cells are fated to give rise to the wing hinge. Consistent with these expression patterns, wg is required for hinge development, but much of the hinge is still formed in the absence of vg (Williams et al., 1993; Neumann and Cohen, 1996a; Klein and Martinez Arias, 1999). These observations raise the following questions: why is wg not expressed at the D/V boundary outside the wing blade and, what are the wg target genes in the hinge that are important for hinge development?

In this paper we show that the gene homothorax (hth), which has been previously shown to be important in the control of Hox function and antennal development (reviewed in Mann and Affolter, 1998), is a wg target gene in the hinge and that hth is required for hinge development. We further show that there is an antagonism between vg and the more proximally expressed genes hth and teashirt (tsh). Finally, we show that in addition to specifying proximal wing structures, hth limits where along the D/V compartment boundary the wing blade can form, thus helping to define the size and position of the wing blade within the disc epithelium.

MATERIALS AND METHODS

Fly stocks

hthP2 (Kurant et al., 1998) is a strong hth hypomorphic allele; we cannot detect Hth protein in hthP2 clones using our chicken anti-Hth antibody, wgP²-Fz, Neumann and Cohen (1996a). fzs1 fzt2 FRT2A, Chen and Struhl (1999).

lacZ reporters: hth-Z: hthP6 (Riechhof et al., 1997); tsh-Z: tsh1 (Fasano et al., 1991); vgŒ-Z (Kim et al., 1996); wg-lacZ: wg-lacZ and CyO, wg-lacZ (Kassiss et al., 1992); fzs-fz (Villano and Katz, 1995).


UAS lines: UAS-GFP (green fluorescent protein); UAS-GFP-hth (8) (Casares and Mann, 1998); UAS-vg (Kim et al., 1996); UAS-tsh (from S. Kerridge); UAS-dTCFDN (Cadigan et al., 1998); UAS>hs-CD2, y+>Nrt-wg (Zecca et al., 1996).

hth- clones

Mitotic recombination was induced by the hsFLP/FRT method (Xu and Rubin, 1993). Collections of embryos of the genotype y hs-FLP, FRT82B hthFRT82B hsCD2, y+ were heat shocked for 20-30 minutes, 37°C, at 0-24, 24-48, 48-72 or 72-96 hours after egg laying (AEL). To mark clones in the adult, we used y or Sh, which in the hinge can mark the allula, costa and tegula. Normal allula and proximal or medial costa were never formed by hth- cells. When hth- tissue was present, the sclerites, axillary cord and radius were also frequently missing or disorganized. Because hth clones in the hinge grow poorly relative to their wild-type twin spots, we also used the Minute (M) technique (Morata and Ripoll, 1975) to provide hth- cells with a growth advantage. For the M experiments, embryonic collections of the genotype y hs-FLP, FRT82B hthhFRT82B Sh M or hs-FLP, FRT82B hthhFRT82B CD2 y+ M were treated as above. In some experiments, fzs-fz or wg-lacZ were introduced into the background. For microscopic examination, specimens were dissected and mounted in Hoyer’s mountant:acetic acid (1:1) (Wieschaus and Nusslein-Volhard, 1986).

Misexpression experiments

The chromosome y hs-FLP, act>hs-CD2>Gal4 was used to drive the expression of: UAS-GFP hth (8) and UAS-tsh in clones. Embryos from the appropriate crosses were collected for 24 hours and heat shocked at 35°C for 30 minutes at different developmental times, as described in the Results.

For Nrt-Wg (or Wg) misexpression we used y hs-FLP; AG11/ UAS>hs-CD2 y+>Nrt-wg (or Wg) (Zecca et al., 1996). Expression of Nrt-Wg protein was detected either directly, by staining for the flu epitope in the protein, or by staining for CD2 after heat-shocking the larvae at 37°C for 30 minutes.

For dTCFDN misexpression: (a) embryos from the cross ptc-Gal4: UAS-GFP x UAS-dTCFDN were collected at 18°C for 2 days, cultured for another 2 days at 18°C and then transferred to 25°C until larval dissection; (b) 24 hour embryo collections from the cross y hs-flp, act>hs-CD2>Gal4 females x UAS-dTCFDN males were heat shocked at 72-96 hours for the induction of clones.

Immunostainings

The antibodies used were: rabbit anti-ß-galactosidase (Cappel); mouse anti-rat CD2 (Serotec); chicken anti-Hth (Casares and Mann, 1998); rabbit anti-Exd (Mann and Abu-Shaar, 1996); rat anti-Tsh (Roder et al., 1992); rabbit anti-Vg (Williams et al., 1991); mouse anti-Wg (4D4; (Neumann and Cohen, 1997)) (Iowa University Hybridoma Bank); mouse anti-Nubbins (Ng et al., 1995) (from Michalis Averof). Appropriate fluorescent secondary antibodies (FITC, Texas Red and Cy-5 conjugated) were from Jackson Laboratories. Imaginal discs were prepared for immunofluorescence and analyzed with a Biorad 1024 confocal system. The mutant tissue in the prospective hinge region of wing imaginal discs was detected by the absence of Hth or cytoplasmic Exd, because hth is required for nuclear Exd (Riechhof et al., 1997; Casares and Mann, 1998; Pai et al., 1998) or by the absence of CD2 or GFP. For induction of CD2 or GFP, larvae were heat shocked for 45 minutes at 37°C and then allowed 45 minutes recovery at 25°C prior to dissection.

Histochemistry

X-gal staining of adult cuticles was performed on flies dissected from their pupal cases as described (Hama et al., 1990). Stained cuticles were mounted in Hoyer’s mountant:acetic acid (1:1) (Wieschaus and Nusslein-Volhard, 1986).

RESULTS

Overlapping patterns of wg and hth expression in the wing hinge

The dorsal portion of the adult second thoracic segment (mesothorax) includes the mesonotum, the major part of the dorsal thorax, and its associated appendage, the wing (Fig. 1A). The distal portion of the wing is referred to as the wing blade. The proximal portion of the wing is referred to as the hinge, and consists of a set of structures that joins the wing blade to the notum. The more distal portion of the hinge is continuous with the wing blade, but contains three identifiable structures,
the costa (Co), the radius (Ra) and the allula (Al) (Fig. 4A). A second, more proximal part of the hinge (or axillary region), is morphologically demarcated from the rest of the wing and consists of several sclerites (Scl), which are mostly devoid of trichomes, and the axillary cord (aCrd) (Bryant, 1978) (Fig. 4A). The tegula (Te), although positioned just anterior to the sclerites, fate maps in the wing disc to a distinct and more dorso-proximal region than these hinge structures, and therefore is not considered a part of the hinge (Figs 1C-E, 4A).

Correspondingly, the distalmost portion of third instar wing discs is referred to as the wing pouch, which will give rise to the wing blade (Fig. 1B-E). Surrounding the wing pouch is a region that will give rise to the hinge and, more proximally, there is a large dorsal territory that will give rise to the mesonotum (mnt) and a thin ventral region that gives rise to the pleura (pl) (Fig. 1B-E). Several genes are known to be expressed in the wing pouch including vestigial (vg), scalloped (sd), nubbin (nub) and Distal-less (Dll), which encode transcription factors, and four-jointed (fj), which encodes a putative secreted factor (Williams et al., 1991; Ng et al., 1995; Villano and Katz, 1995; Brodsky and Steller, 1996; Kim et al., 1996; Zecca et al., 1996; Neumann and Cohen, 1997) (Figs 1F, 5A,G and data not shown). Dll and vg are both wg target genes in the wing blade and are expressed in a graded manner, with peak levels at the D/V boundary and gradually lower levels in more dorsal and ventral cells (Fig. 1F,G and data not shown) (Zecca et al., 1996; Neumann and Cohen, 1997). vg is also expressed outside the wing pouch, along the D/V boundary in the posterior compartment and, in the anterior compartment, in a strong dorso-ventral patch of cells close to the hinge (Fig. 5A) (Williams et al., 1991, 1994).

wg is expressed along the D/V compartment boundary within the wing blade and in two concentric rings that surround the wing blade region (Kassis et al., 1992; Couso et al., 1993; Williams et al., 1993; Neumann and Cohen, 1996a) (Fig. 1C,D). The rings of wg expression have been fate mapped to the adult hinge (Neumann and Cohen, 1996a) and, using a wg-lacZ reporter gene, they map within the hinge as follows: the outer wg ring (OR) maps to the proximal hinge (Fig. 1D,J), and the inner wg ring (IR) stains structures in the distal hinge, including the medial costa (mCo), distal radius (dRa) and part of the allula (Al) (Fig. 1D,J) (see also Neumann and Cohen, 1996a). hth is also highly expressed in the wing hinge region of third instar wing discs, straddling both wg rings (Fig. 1C,E). Using a hth-lacZ reporter gene, hth expression maps to the same structures in the adult hinge as does wg (Fig. 1K). In late third instar wing discs, teashirt (tsh), which codes a Zn-finger transcription factor (Fasano et al., 1991), is strongly expressed in cells that are more proximal than hth-expressing cells, although low levels of tsh and hth overlap in the proximal hinge region (Fig. 1H,I). Consistent with this expression pattern, tsh-expressing cells fate map in the adult to the axillary sclerites and pleura (Fig. 1L).

The temporal progression of hth expression in the wing disc
To understand the evolution of these expression patterns, we examined hth, wg and tsh expression at early and intermediate stages of wing disc development. Early in second instar, before wg expression initiates, all wing disc cells express hth (Fig. 2A). Weak vg expression is also observed throughout the wing disc at this early stage (Williams et al., 1993), suggesting that wing disc cells at this stage are not fated to be either proximal or distal. Once wg is activated, hth is repressed in the wg-expressing cells but remains active in all non-wing blade cells until mid-third instar (Fig. 2B-E). Soon after wg expression initiates, wg and vg are activated along the newly formed D/V boundary, and nub is activated in the wing pouch (Ng et al., 1995; Kim et al., 1996; Zecca et al., 1996; Neumann and Cohen, 1997) (Fig. 2F-H and data not shown). From early second instar through early third instar, tsh is expressed in the same pattern as hth (Fig. 2I and data not shown). As the wing disc grows in size these expression patterns essentially stay constant until the mid-third instar stage, when hth is upregulated in the hinge and peak levels of tsh become restricted to more proximal regions of the disc (Figs 2E, 1H).

These results indicate that, in the mid-second instar, hth and tsh expression are turned off in the presumptive wing blade and are kept off in this region of the disc for the remainder of development. Further, these results show that late in development, hth is upregulated in the hinge region and tsh is downregulated in this domain. Below, we provide evidence that the upregulation of hth in the hinge is due to wg signaling.

hth is a wg target gene in the hinge
The overlap between wg and high levels of hth in the hinge region (Fig. 1C-E) suggested that wg might play a role in activating hth in this region of the wing disc. We carried out four experiments to test this idea. In the first, we examined hth expression in discs in which the wg signaling cascade was compromised due to the expression of a dominant negative form of dTCF, a downstream transcription factor in the wg signal transduction pathway (Cadigan et al., 1998). Expression of dominant negative dTCF (dTCF<sup>DN</sup>) using ptc-Gal4 resulted in the repression of hth in the hinge (Fig. 3A,B). Similar results were obtained when flip-out clones expressing dTCF<sup>DN</sup> were generated in the hinge between 72 and 96 hours of development (data not shown, see Materials and Methods). A second piece of evidence supporting a role for wg in the upregulation of hth is our finding that in wg<sup>quad</sup>-<sup>tg</sup> mutant discs, in which wg expression is specifically reduced in the IR of third instar discs (Neumann and Cohen, 1996a), hth expression is no longer upregulated (data not shown). Third, we generated clones of cells doubly mutant for frizzled1 and frizzled2 (fz1<sup>–</sup>fz2<sup>–</sup>), which are required for the reception of the Wg signal (Bhat, 1998; Bhanot et al., 1999; Chen and Struhl, 1999). fz1<sup>–</sup>fz2<sup>–</sup> clones showed a cell-autonomous loss of hth expression (Fig. 3C-E). These findings suggest that wg signaling, mediated by the Frizzled family of receptors, is required for high levels of hth expression in the hinge region of wing discs.

In a fourth experiment, we tested if ectopic expression of wg could trigger the expression of hth in more proximal regions of the wing disc, where hth levels are usually low and tsh levels are high (Fig. 1H,I). Based on the expression patterns of tsh and hth in third instar discs (Fig. 1H,I), we predicted that, if induced by wg, hth would also repress tsh. We generated flip-out clones of wild-type (secreted) Wg or of a membrane-tethered, and therefore non-diffusible, form of Wg, Nrt-Wg (Zecca et al., 1996), and monitored the expression of wg, hth and tsh. Expression of either Wg or Nrt-Wg in clones in the notum (just dorsal to the hinge region) non-autonomously induced high levels of hth and repressed tsh, recapitulating the
situation found in the wild-type hinge (Fig. 3F-I and data not shown). In contrast to clones induced in the notum portion of the disc, Nrt-Wg was never observed to activate hth in the wing pouch. Instead, activation of the Wg pathway in the wing pouch induces higher levels of vg expression (Zecca et al., 1996; Neumann and Cohen, 1997).

These data suggest that hth is a wg target gene in the wing hinge. In addition, they suggest that, in response to wg, cells in the wing pouch are biased in favor of activating vg, whereas in more proximal positions, induction of hth is favored.

**hth is required to form the hinge**

In order to examine the role that hth plays in the wing disc we studied the consequences of both removing hth activity and ectopically expressing hth during development. To remove hth activity we generated by mitotic recombination hth– clones. In hth– clones in the adult that are within the hinge region, hinge structures are severely disrupted or absent (Fig. 4A,B) (see Materials and Methods). Specifically, the radius, axillary cord, sclerites, proximal and medial costa and allula do not form in the absence of hth. In contrast, the tegula and distal costa are formed in the absence of hth. Similar phenotypes were observed in the absence of extradenticle (exd)
function (Gonzalez-Crespo and Morata, 1995; Rauskolb et al., 1995), consistent with the role that hth plays in the nuclear localization of the exd gene product (Rieckhof et al., 1997; Pai et al., 1998).

Fig. 3. hth is a wg target gene in the hinge. (A,B) The hinge region of a wing disc in which dTCF<sup>DN</sup> and GFP were coexpressed using ptc-Gal4, and stained for GFP (red) and Hth (green). (C-E) Hinge region in which fz<sup>1</sup>-fz<sup>2</sup> mutant clones are present (marked by the absence of GFP staining; red) and stained for Hth (green). (F-I) AG11-Gal4; UAS<sup>+</sup>-Nrt-wg flip-out clones in the mesonotum, just dorsal to the hinge, stained for the flu epitope of Nrt-Wg (white) (Zecca et al., 1996), Hth (green) and Tsh (red). In the clone closer to the hinge (arrow), Nrt-Wg can be detected in only one cell, but hth expression is observed in many cells. The strong stripe of Hth expression at the bottom of these panels is in the OR of the hinge; dorsal is up.

Fig. 4. hth is required for hinge formation and to limit the size of the wing. (A) High magnification view of a wild-type wing hinge. Scl, sclerites; aCrd, axillary cord; Co, costa; Ra, radius; Al, allula; Te, tegula. (B) Wing hinge of a fly in which hth<sup>–</sup> clones were induced during second instar. The medial Co and part of the radius are absent; their expected positions are indicated by the filled and open arrowheads, respectively. The aCrd is absent and replaced by disorganized tissue (asterisk). y<sup>–</sup> bristles in the distal Co and Te (arrows; both wild type) indicate the presence of hth<sup>–</sup> tissue. (C,D) hth misexpression in the dorsal wing, driven by the 1096-Gal4 driver line, reduces the wing to a rudiment (Fig. 4C,D,H). Driving hth expression with the 1096-Gal4 driver line, which is expressed primarily in the dorsal compartment of the wing disc, results in winglets that, on the dorsal surface, have three types of tissue (Fig. 4C,D): (1) an apparent extension of distal hinge tissue that is similar to the radius (by the density and size of trichomes), (2) an unpigmented transparent cuticle that may be sclerital tissue, and (3) a small amount of D/V boundary tissue. We interpret this phenotype as resulting from a repression of wing development and a partial transformation of wing into radius and sclerite tissues. Together with the loss-of-function phenotypes, these data suggest that hth is required for hinge development and, in some contexts, is sufficient to specify hinge structures.

hth limits where along the D/V compartment boundary the wing will develop

If larger hth<sup>–</sup> clones are induced with the Minute (M) technique during the first or early second instar stages, large overgrowths of wing blade tissue are frequently produced in place of the hinge (Fig. 4E). These overgrowths are located posteriorly, but
can contain posterior (indicated by double row wing margin bristles) as well as anterior (indicated by the presence of vein 3-type sensilla campaniformia) wing tissue (Fig. 4G). All of the overgrowths we observed in adult flies include an ectopic posterior wing margin, indicating the presence of a D/V compartment boundary in the overgrown tissue. These overgrowths often contain wild-type (hth+ cells) that had been induced to form wing (Fig. 4F). In contrast, hth– clones within the wing blade are normal, and the mesonotum develops almost normally in the absence of hth, with only minor alterations in the pattern of bristles, even when most of the tissue is mutant (Fig. 4E). In clones that delete the hinge, wing and notum tissues appear to mix.

Consistent with these adult phenotypes, we also observe large tissue overgrowths in wing imaginal discs containing hth–M+ clones. When observed, these overgrowths are of two types: ventral overgrowths that do not express wing pouch markers (nub, vg, fj-lacZ and Dll) (Fig. 5E,H and data not shown) and posterior overgrowths that do express these markers (Fig. 5B,D,E,H,I). The second class of overgrowths straddle the D/V compartment boundary, as marked by an extended stripe of Wg expression (Fig. 5C) and by expression of a vg boundary enhancer-lacZ reporter gene (vgBE-lacZ;...
Refinement of gene expression patterns. (A,B) hth- clones, induced in a wg-lacZ background (arrowheads), in the dorsal IR of the hinge stained for CD2 (green) and Wg-lacZ (red). The mutant tissue is outlined in white. Compared to the levels in wild-type cells (arrowheads), Wg-lacZ levels are lower in hth- cells. (C,D) actin>CD2>Gal4; UAS:GFP-hth flip-out clones in the notum region just dorsal to the hinge, stained for Tsh (red) and GFP-Hth (green). The arrows point to examples of GFP-Hth-expressing clones with lower levels of Tsh.

Williams et al., (1994) (data not shown). As with the adult phenotypes, the disc overgrowths can in part be composed of wild-type tissue (Fig. 5H,I). The ectopic expression of wg may account for the non-autonomous transformations observed in adults and discs containing hth- clones. In contrast to clones that straddle the D/V boundary, hth- clones that are restricted to the dorsal compartment do not overgrow (Fig. 5E,F). However, even in these clones the absence of wild-type hinge tissue is apparent because of the lack of folds typically found in this region of the epithelium (Fig. 5B,D-F). Occasionally, a small expansion of wing blade markers was observed in anterior hth- clones that straddle the D/V boundary, but these overgrowths are always smaller than those in the posterior compartment, even when most of the anterior compartment is mutant (Fig. 5B,D).

When hth is ectopically expressed at the D/V boundary with the vg-boundary enhancer-Gal4 driver line (vgBE-Gal4) (Simmonds et al., 1998), the wings are dramatically reduced in size, and the formation of the wing margin is suppressed (Fig. 4H). However, unlike the winglets induced by 1096-Gal4 (Fig. 4C,D), these winglets do not show an expansion of hinge-like tissue, but instead appear to maintain their wing blade identity. The hinge region appears largely intact in these wings. Consistent with the adult phenotype, vgBE-Gal4; UAS-GFP-hth wing discs have very small wing pouches (Fig. 6A). When these discs were stained for Vg, we found that Vg could only be detected at or very close to the D/V boundary (Fig. 6B). Thus, expression of Hth driven by vgBE-Gal4 results in the suppression of vg expression in the wing pouch, consistent with its reduced size, but does not block vg expression at the D/V boundary. In addition, we found that expression of Hth driven by vgBE-Gal4 also reduced, but did not eliminate, wg expression at the D/V boundary (Fig. 6C). When expressed at the D/V boundary Hth was also able to repress expression of cut, which is a downstream target of the Notch pathway (de Celis et al., 1996; Michelli et al., 1997; Neumann and Cohen, 1998) (Fig. 6D,E). However, in contrast to its ability to interfere with wg expression when expressed at the D/V boundary, when Hth was expressed in clones that did not intersect the D/V boundary, no repression of vg was observed (data not shown). Thus, for hth to repress vg in the wing blade it must be expressed at the D/V boundary, suggesting that hth inhibits an activity present at the D/V boundary that is required for vg expression in the wing pouch. Because Hth represses cut and partially represses wg, both Notch target genes, these data suggest that Hth interferes with a subset of Notch functions at the D/V boundary.

tsh collaborates with hth to block wg expression at the D/V boundary

When expressed at the D/V boundary, Hth is able to reduce wg and vg expression, and block cut expression. These expression patterns begin to approach those at the wild-type D/V boundary outside the wing pouch in the posterior compartment, where vg is expressed but wg and cut are not (Fig. 1B) (data not shown). The residual wg expression observed in the wing blades of vgBE-Gal4; UAS-GFP-Hth discs suggests that other factors may be required to completely eliminate wg expression at the D/V boundary. One such factor may be tsh, which is coexpressed with hth throughout most of wing disc development. To test a potential role for tsh, we ectopically expressed Tsh, both in clones and along the D/V compartment boundary, and examined the effects on hth, vg and wg.

Surprisingly, we found that tsh is a potent inducer of hth in the wing blade. When expressed in clones, tsh strongly induced hth expression in a cell-autonomous manner (Fig. 6F-I). These clones had a strong tendency to sort away from the neighboring wild-type cells (Fig. 6K,L). When we examined Vg in these clones, we found that it was strongly repressed (Fig. 6F-J). Thus, unlike Hth, the combination of Tsh plus Hth is able to repress vg in the wing blade, even when expressed away from the D/V boundary.
When expressed at the D/V boundary using the vgBE-Gal4 driver, tsh also induced hth and eliminated wing blade development (data not shown). wg expression was completely repressed in these wing blades (Fig. 6M-O). Strikingly, although vg expression was absent in the remaining wing pouch, it was still expressed at the D/V boundary (Fig. 6N). These expression patterns (vg, on; wg, off) are normally observed at the D/V boundary outside the wing pouch in the posterior compartment. These results suggest that the combination of tsh plus hth blocks wg expression at the D/V boundary. In addition, because removing hth alone is sufficient to derepress wg at the D/V boundary (Fig. 5C), these results suggest that tsh is not sufficient to repress wg, and that hth is necessary.

Refinement of the gene expression patterns
Between the early third and late third instar stages, three changes in the expression patterns of these genes are apparent. The first change is with hth, which is strongly upregulated in the hinge due, at least in part, to activation by wg (see above). The second notable change is with wg expression. For most of development, wg (as monitored with an anti-Wg antibody) is weak at the edge of the growing wing blade field, but is upregulated in the hinge in mid-third instar discs (Fig. 2). This increase in wg expression in the hinge requires hth. hth– clones in the hinge, induced during the second instar larval stage, resulted in a reduction of wg expression, as monitored by the wg-lacZ reporter gene (Fig. 7A,B) and in the levels of Wg protein (not shown). hth– clones such as these that are restricted to the dorsal compartment do not derepress wg or cause wing overgrowths (Fig. 5E,F). These data suggest that hth is required to induce the late upregulation of wg expression in the hinge region of third instar discs.

A third change that occurs late in development is the downregulation of tsh in the hinge. Although hth and tsh are coexpressed throughout most of wing disc development (Fig. 2J), by the end of the third instar stage, high levels of hth and tsh expression do not overlap, and peak levels of tsh are observed in cells that are more proximal than those expressing peak levels of hth (Fig. 1H,I). We suggest that this change in expression pattern is because high levels of hth downregulate tsh. Consistent with this idea, flip-out clones expressing GFP-Hth in the tsh domain show a cell-autonomous downregulation of tsh expression (Fig. 7C,D; see also Fig. 3H,I). In addition, we also observed the upregulation of tsh expression in hth– clones in more dorsal regions of the notum (data not shown).

DISCUSSION

A role for hth in conferring proximal wing identities
The wing of Drosophila consists of two major subdomains along the P-D axis: a more distal region, called the wing blade, and a more proximal region, called the hinge. The development of both proximal and distal portions of the wing require the activity of wg. In the wing blade, an important wg target gene is vg (Williams et al., 1991). Here we show that hth is required for the specification of the hinge and that, like vg in the wing blade, hth is a wg target gene.

The requirement for hth in hinge development is supported by both loss-of-function and ectopic expression experiments. For those structures that can be marked with the y marker gene (the allula, the costa and the tegula), we never observed hth– cells forming a normal allula, proximal costa or medial costa. In addition, hth– clones were also frequently associated with the absence of the sclerites and proximal radius. These effects are consistent with the phenotypes seen upon ectopic expression of Hth in the wing with the 1096-Gal4 driver line, where we observed an expansion of hinge structures resembling the sclerites and radius. Together with the high levels of hth expression in the prospective hinge region of the wing disc, these results demonstrate that hth is necessary for the proper development of the hinge. As expected from the association of the hth and exd gene products (Rieckhof et al., 1997; Abu-Shaar et al., 1999), these effects on hinge development are similar to those reported previously for exd (Gonzalez-Crespo and Morata, 1995; Rauskolb et al., 1995).

Activation of hth and vg by wg
The experiments presented here demonstrate that wg induces hth expression in the hinge, and previous results demonstrate that vg is upregulated by wg in the wing pouch (Zecca et al., 1996; Neumann and Cohen, 1997) (Fig. 8). These observations raise the question of how the same signaling molecule induces the expression of two different target genes at different positions along the P-D axis. Our results are consistent with the idea that, in wild-type discs, some property of the wing blade makes these cells predisposed to activate vg instead of hth. This property may in part be due to vg itself, which is independently activated by Notch at the D/V boundary. Because vg activates its own expression (Halder et al., 1998), cells that previously expressed vg may be biased in favor of activating vg in response to wg (Klein and Martinez-Arias, 1998, 1999). In cells that have not previously expressed vg, such as in the hinge, this bias may not exist, allowing wg to activate other targets such as hth.

Surprisingly, we found that a membrane-tethered and therefore non-diffusible form of Wg, Nrt-Wg, could cell-non-autonomously induce hth expression in the notum. In contrast, in the wing pouch, Nrt-Wg activated vg only in Nrt-Wg-expressing cells and immediately adjacent cells (Zecca et al., 1996). These findings raise the possibility that, in the notum, Nrt-Wg may be activating the expression of another diffusible factor, whose identity is unknown. The cell-autonomous loss of hth expression in fz; lz– clones suggests that this factor may be a Wnt. One possibility is that wg, itself, is induced by Nrt-Wg in the notum.

A role for hth and tsh in blocking wing blade development at the D/V compartment boundary
In addition to the disruption of proximal wing structures, a second, and more dramatic, phenotype resulting from the loss of hth function was the formation of large overgrowths of the wing blade in adults and ectopic wing pouches in imaginal discs. These overgrowths occur posteriorly, and always contain both dorsal and ventral cells. From this observation we infer that for ectopic wing blades to form, hth function must be eliminated from both sides of the D/V boundary. This can occur if the clone is generated early, before the D/V lineage restriction is formed, or later, if two hth– clones arise on either side of the D/V boundary. In such overgrowths, we invariably observed the ectopic expression of wing pouch markers, such
raise the question of what step in wing pouch development continues to repress, even in the absence of posterior hth – the D/V boundary in the posterior compartment. Therefore, a latent potential of the D/V boundary in the posterior compartment to initiate wing blade development. Thus, there are factors that repress vg expression in the anterior compartment. We suggest that these factors continue to repress vg, even in the absence of hth, and that without vg expression a wing blade cannot grow (Kim et al., 1996).

The wing blade overgrowths observed in the P compartment raise the question of what step in wing pouch development hth might block. In wild-type discs, the growth of wing blade is thought to initiate at the D/V boundary, and require the activities of the wg, Notch and EGF pathways (Diaz-Benjumea and Garcia-Bellido, 1990; Couso et al., 1993; Williams et al., 1993, 1994; Diaz-Benjumea and Cohen, 1995; Ng et al., 1996; Nagaraj et al., 1999). Although all three of these pathways have been implicated in wing development, they appear to play distinct roles. wg is required for the initiation of wing development (Couso et al., 1993; Williams et al., 1993, 1994; Ng et al., 1996), but when this pathway is ectopically activated it does not induce the wing pouch to grow larger than normal (Zecca et al., 1996; Neumann and Cohen, 1997; Klein and Martinez-Arias, 1998; Nagaraj et al., 1999). In contrast, activation of either the Notch or EGF pathways does induce additional growth of the wing pouch (Diaz-Benjumea and Cohen, 1995; Klein and Martinez-Arias, 1998; Nagaraj et al., 1999). Strikingly, when we ectopically expressed hth at the D/V boundary, it inhibited vg activation in the wing pouch, but not at the D/V boundary. hth expression at the D/V boundary also partially repressed wg expression and completely blocked cut expression. Because cut and wg are both activated by Notch at the D/V boundary, these results suggest that hth is able to block, at least partially, Notch’s ability to activate some of its targets at the D/V boundary (Fig. 8). Importantly, however, hth does not block all Notch activity because vg expression at the D/V boundary, which is also activated by Notch, was still observed in the presence of Hth.

Additional experiments presented here suggest that tsh collaborates with hth to interfere with Notch’s ability to activate wg at the D/V boundary (Fig. 8). During wild-type wing disc development, both hth and tsh are coexpressed in all non-wing blade cells and, at least in the posterior compartment, the D/V boundary expresses vg but not wg. Consistent with these wild-type expression patterns, the combination of Hth plus Tsh was sufficient to completely block wg expression at the D/V boundary in the wing blade. In contrast, vg was still expressed at the D/V boundary in the presence of both Hth and Tsh. We suggest that the repression of wg by Hth and Tsh represents a normal function of these two proximally expressed transcription factors. The results further suggest that hth is necessary for this repression, because wg is derepressed in hth- clones that straddle the D/V boundary. We note that we have not yet examined the effects of removing tsh function because the close centromere linkage of tsh complicates the generation of tsh- clones. However, this is clearly an important experiment that must eventually be carried out to fully address the role that tsh plays in wing disc development.

Conclusions

In summary, these experiments demonstrate that hth plays at least two roles in wing development. First, hth is required to limit where along the D/V boundary the wing blade will form. We suggest that hth carries out this function at least in part by interfering with Notch’s ability to activate wg. In addition, it is possible that hth also interferes with Wg’s ability to activate the vg quadrant enhancer. These results further suggest that, in wild-type wing discs, hth works together with tsh to block wing blade development. Second, hth is required for the identity of the proximal wing (the hinge), because in the absence of hth function, the hinge cannot form. It is of interest that both of these functions have parallels in leg development, where hth is also required for proximal appendage identities, and also interferes with the activities of signaling pathways (Gonzalez-Crespo and Morata, 1995; Rauskolb et al., 1995; Abu-Shaar and Mann, 1998; Gonzalez-Crespo et al., 1998). In the future, it will be important to gain an understanding of the mechanism(s) by which hth interferes with signaling pathways, and to determine if the mechanisms used in leg development are the same as those used in wing development.

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