Control of growth and patterning of the *Drosophila* wing imaginal disc by EGFR-mediated signaling

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Accepted 7 December 2001

SUMMARY

The subdivision of the *Drosophila* wing imaginal disc into dorsoventral (DV) compartments and limb-body wall (wing-notum) primordia depends on Epidermal Growth Factor Receptor (EGFR) signaling, which heritably activates *apterous* (*ap*) in D compartment cells and maintains Iroquois Complex (Iro-C) gene expression in prospective notum cells. We examine the source, identity and mode of action of the EGFR ligand(s) that specify these subdivisions. Of the three known ligands for the *Drosophila* EGFR, only Vein (Vn), but not Spitz or Gurken, is required for wing disc development. We show that Vn activity is required specifically in the dorsoproximal region of the wing disc for *ap* and Iro-C gene expression. However, ectopic expression of Vn in other locations does not reorganize *ap* or Iro-C gene expression. Hence, Vn appears to play a permissive rather than an instructive role in organizing the DV and wing-notum segregations, implying the existence of other localized factors that control where Vn-EGFR signaling is effective. After *ap* is heritably activated, the level of EGFR activity declines in D compartment cells as they proliferate and move ventrally, away from the source of the instructive ligand. We present evidence that this reduction is necessary for D and V compartment cells to interact along the compartment boundary to induce signals, like Wingless (Wg), which organize the subsequent growth and differentiation of the wing primordium.

Key words: EGFR signaling, Pattern formation, Wing imaginal disc, *Drosophila*, Compartment boundary, Organizing activity

INTRODUCTION

During development, the wing imaginal disc is subdivided into distinct anteroposterior (AP), dorsoventral (DV) and wing-notum (limb-body wall) primordia. The AP and DV subdivisions constitute developmental compartments that are established early via the heritable activation of the genes *engrailed* (*en*) and *apterous* (*ap*) (for reviews, see Blair, 1995; Dahmann and Basler, 1999; Lawrence and Struhl, 1996). The state of activity of these genes, whether ‘on’ or ‘off’, then governs the behavior of cells in each compartment, generating an abrupt cellular interface that is both a barrier to cell mixing and the source of morphogens that organize the development of both compartments. By contrast, the wing-notum subdivision is not a compartmental segregation (Diez del Corral et al., 1999). Instead, presumptive notum cells are distinguished from wing cells by the continuous input of signals that maintain the activity of Iroquois Complex (Iro-C) genes (Wang et al., 2000; Zecca and Struhl, 2002). As a consequence, cells can switch between being of prospective notum or wing type as they move in or out of the range of these signals.

We show in the accompanying paper (Zecca and Struhl, 2002) that both the DV and wing-notum subdivisions are governed by Epidermal Growth Factor Receptor (EGFR) signaling, via the early heritable activation of *ap* and by the continuous maintenance of Iro-C gene expression. We now examine the identity and source of the signal(s) responsible for controlling both segregations, as well as the mechanisms by which these signals control the overall growth and patterning of the wing disc.

Our results implicate Vein (Vn), one of three known ligands for the EGFR, in the activation of both *ap* and Iro-C gene expression, and indicate that it is normally provided by cells located in the dorsoproximal region of the disc. However, ectopic expression of Vn in other regions of the disc does not induce ectopic *ap* or Iro-C expression, nor is it able to restore dorsal expression of these genes in the absence of endogenous Vn activity. These results indicate that Vn functions as a permissive rather than an instructive factor in establishing the DV and wing-notum subdivisions. For example, Vn may normally have to be processed by, or act in conjunction with, other dorsally expressed factors to generate the instructive EGFR ligand. Alternatively, Vn-EGFR signaling may be inhibited by factors expressed in more ventrally situated cells.

Our results also suggest an unexpected mechanism for controlling growth and patterning of the wing. Although both the *ap* and Iro-C genes are first expressed within overlapping dorsal domains of the early wing disc, the boundary of *ap* expression then shifts ventrally relative to that of Iro-C.
expression, indicating that the level of EGFR signaling declines in D compartment cells as they proliferate and move out of the range of the instructive EGFR signal. We find that this reduction is essential for D and V compartment cells to interact to form a new and stable source of organizing signals, such as the morphogen Wingless (Wg), which control subsequent wing development.

MATERIALS AND METHODS

Ectopic expression studies
Clones of cells that ectopically express Vn, Rho, Ap or activated Ras (RasV12) were generated using the FLP-out and Gal4/UAS techniques, as described elsewhere (Zecca and Struhl, 2002). Ap-, Rho- or Vn-expressing clones were induced by using Tubα1>flu-GFP, y>Gal4 transgene in combination with either UAS-ap (O’Keefe and Thomas, 2001), UAS-rho (Golebo et al., 1996) or UAS-vn (Schnepf et al., 1998) transgenes. RasV12-expressing clones were generated using a Tubα1>Gal80, y>Gal4 transgene, together with the transgenes UAS-RasV12 (Karim and Rubin, 1998) and UAS-GFP (Zecca and Struhl, 2002). Clones were either induced in wild-type or vn1/2vnΔddRR (Schnepf et al., 1996) larvae carrying appropriate lacZ reporter transgenes (see below for detailed genotypes), and marked by the loss (Ap, Rho, Vn) or gain (RasV12) of GFP expression. To generate clones during first instar, larvae were heat shocked 24-48 hours after egg laying (AEL) for 30 minutes at 36°C to obtain just one clone on average per disc. To generate clones during first instar, larvae were heat shocked 24-48 hours after egg laying (AEL) for 30 minutes at 35°C. To obtain widespread ectopic expression of Vn, larvae were heat shocked 24-48 hours AEL for 20 minutes at 35°C. To obtain widespread ectopic expression of Vn, larvae were heat shocked 24-48 hours AEL for 60 minutes at 37°C.

The genotype for vn mutant larvae are as follows: (i) y w hsps70-flp; ap-lacZ/+; Tubα1>flu-GFP, y>Gal4 UAS-rho vnΔddRR. (ii) y w hsps70-flp; Tubα1>flu-GFP, y>Gal4 UAS-rho vt1/2vnΔddRR mirr-lacZ. (iii) y w hsps70-flp; UAS-vn (or UAS-ap); Tubα1>flu-GFP, y>Gal4 vnΔddRR mirr-lacZ. (iv) y w hsps70-flp UAS-GFP; UAS-RasV12+/+; Tubα1>Gal80, y>Gal4 vt1/2vnΔddRR mirr-lacZ.

lacZ reporter lines and antisera staining
lacZ reporter lines and antisera used are described in the accompanying paper (Zecca and Struhl, 2002), except for wgcn11 (Kassis et al., 1992), a wg-lacZ reporter used only here (Fig. 3C). Imaginal discs from wandering third instar larvae were fixed and stained following standard protocols.

RESULTS

The EGFR ligand Vn is necessary, but not sufficient, to organize ap and Iro-C expression
Three ligands for the Drosophila EGFR have been identified to date, Spitz, Vein and Gurken (Rutledge et al., 1992; Neuman-Silberberg and Schubach, 1993; Schnepf et al., 1996). Of these, only Vein (Vn) is known to be required for early wing disc development and, strikingly, it is expressed in the presumptive dorsal region of the disc, where ap and Iro-C gene expression are first detected during the second larval instar (Simcox et al., 1996; Wang et al., 2000).

To assess a possible role for Vn in organizing ap and Iro-C gene expression, we first asked whether ectopic Vn expression altered the normal domains of ap and the Iro-C gene mirror (mirr). Clones of cells expressing Vn under Gal4/UAS control were induced during the first larval instar (Materials and Methods). However, we could not detect any effect of such forced Vn expression on either ap and mirr-lacZ expression, even when multiple clones were scattered throughout a single disc (data not shown).

We next examined the consequences of ectopically expressing Vn in wing imaginal discs devoid of endogenous vn activity. vn mutant wing discs proliferate very poorly, if at all and do not express either ap or Iro-C genes (Simcox et al., 1996; Wang et al., 2000). However, vn mutant discs that contained multiple Vn expressing clones appeared similar to wild-type discs in size as well as the patterns of ap and mirr-lacZ gene expression (Fig. 1A). Moreover, single Vn expressing clones could confer at least partial (Fig. 1B), if not extensive (Fig. 1D), rescue of growth as well as ap and mirr-lacZ expression in vn mutant discs.

Although Vn-expressing clones have the capacity to rescue normal development in vn mutant discs, rescue was only observed when clones were located in or near the prospective dorsal region of the disc. Of the greater than 100 rescued vn mutant discs recovered, all but one contained Vn-expressing clones that contributed to the rescued D compartment, including a subset of 12 discs that contained only a single clone of Vn-expressing cells (as in Fig. 1B). The one exceptional disc (Fig. 1C) contained two Vn-expressing clones, a small clone located at the dorsoproximal edge of the disc and a larger clone located laterally and close to the rescued D compartment.

Although Vn must be expressed dorsally to rescue mirr-lacZ and ap expression in vn mutant discs, Vn-expressing cells exert their rescuing activity non-autonomously. mirr-lacZ gene expression was restored not only in Vn-expressing cells but also in nearby, non-expressing cells over a range of at least 5-10 cell diameters (Fig. 1B-D). ap expression was also rescued in non-expressing cells, although in this case, the boundaries of ap expression could extend at least 20-30 cell diameters away from the clone border (Fig. 1B,D). However, as we show in the accompanying paper (Zecca and Struhl, 2002), ap expression depends on EGFR signaling only during early wing disc development, in contrast to Iro-C gene expression, which requires continuous EGFR input. Hence, the boundaries of ap expression in the mature disc do not reflect the direct response of cells to Vn activity at this late stage. Instead, we infer that Vn-expressing clones rescue ap expression at shorter range during early wing disc development, committing the descendants of these cells to continue to express ap, even if they subsequently move out of the range of Vn-dependent signaling.

In summary, Vn expression in the dorsal portion of the disc is necessary for the normal activation of the ap and mirr-lacZ genes. However, ectopic expression of Vn in the ventral portion of the disc does not induce ectopic ventral expression of either gene, nor does it restore their normal, dorsal expression in the absence of endogenous vn gene function. We conclude that Vn serves a permissive rather than instructive role in the activation of both ap and the Iro-C genes (see Discussion).

Ectopic activity of the EGFR ligand Spitz can organize ap and Iro-C gene expression
The spitz (spi) gene is expressed in most or all ectodermal cells, yielding a membrane bound, but inactive, EGFR ligand (Rutledge et al., 1992; Sturtevant et al., 1993). This inert form of Spi is then processed to generate the active, secreted EGFR ligand, an event that is promoted by the transmembrane protein.
Rhomboid (Rho) (Schweitzer et al., 1995; Golembo et al., 1996; Bang and Kintner, 2000) and normally occurs only late in wing disc development, when Rho is first expressed (Sturtevant et al., 1993; Simcox, 1997; Guichard et al., 1999; Culi et al., 2001). To assess whether localized production of an EGFR ligand is sufficient to organize the normal patterns of ap and Iro-C gene expression in the wing disc, we generated marked clones of Rho-expressing cells to create ectopic sources of active Spi during early wing disc development.

Initially, we examined the effects of Rho-expressing clones generated during the first larval instar in otherwise wild-type discs. Unlike clones of Vn-expressing cells, which have no effect on normal wing disc development, clones of Rho-expressing cells can cause dramatic reorganization, including the formation of ectopic D compartments (Fig. 2A) and ectopic notum primordia (Fig. 2B), or the abnormal expansion of the D compartment (Fig. 2B,C). Rho-expressing clones were typically associated with ectopic ap expression that can extend at least 10-20 cell diameters beyond the clone borders (Fig. 2B,C), whereas the non-autonomous effects on mirr-lacZ expression were more restricted, extending, at most, 5-10 cell diameters into neighboring, wild-type tissue (Fig. 2B). As already discussed for the rescuing activity of Vn-expressing clones, we attribute this difference in the range of the ap and Iro-C gene responses to the distinct early and continuous roles for EGFR signaling in directing their expression.

We next examined the consequences of ectopically expressing Rho in vn– discs. Like Vn-expressing clones, Rho-expressing clones induced in first instar larvae were able to stimulate proliferation in vn mutant discs. Moreover, Rho-expressing clones within such ‘rescued’ discs were invariably associated with the short-range induction of mirr-lacZ expression as well as the formation of large, sharply bounded, patches of ap-lacZ expression extending many cell diameters beyond the clone borders (Fig. 2D,E; see also Fig. 3B). When located in the dorsoproximal sector of the disc, such clones could rescue most aspects of growth and patterning in the absence of endogenous vn function (Fig. 2E). However, Rho-expressing clones stimulated growth as well as ap and Iro-C gene expression irrespective of their position within vn mutant discs (e.g. Fig. 3B). In this respect, they differ from Vn-expressing clones, which were capable of rescuing growth and the expression of both genes only when located dorsally.

Thus, the behavior of Rho-expressing clones provides a proof of principle that localized production of an active EGFR ligand is sufficient to organize the subdivision of the wing disc into DV compartments and wing-notum primordia. vn mutant discs apparently lack the capacity to generate such an instructive EGFR ligand, and hence fail to develop unless provided with exogenous Vn or Rho activity.

**Reduced EGFR/Ras signaling is required for the DV compartmental interface to acquire organizer activity**

During normal development, ap and the Iro-C genes are initially activated in overlapping dorsoproximal domains in response to EGFR/Ras signaling (Williams et al., 1993; Diez del Corral et al., 1999; Wang et al., 2000). However, the domain of ap expression then expands ventrally relative to that of Iro-C gene expression, into a region of low EGFR/Ras signaling, and the DV compartment boundary becomes a stable source of Wingless (Wg) and other signals (Williams et al., 1994; Diaz-Benjumea and Cohen, 1995; Fleming et al., 1997; Panin et al., 1997), which organize the subsequent growth and differentiation of the wing primordium (Zecca et al., 1993; Simcox, 1997; Guichard et al., 1999; Culi et al., 2001). The inset shows a vn– mutant disc at the same magnification. (A) vn– disc containing multiple Vn-expressing clones. The size and shape of the disc are close to normal, as are the patterns of Ap and mirr-lacZ expression. (B) vn– disc containing a single dorsolaterally positioned Vn-expressing clone (arrow). The clone is associated with a long-range, non-autonomous rescue of Ap expression, but only a short-range rescue of mirr-lacZ expression in a restricted lateral region of the presumptive wing hinge where it is normally expressed. The notum primordium is absent. (C) vn– disc containing a laterally positioned clone of Vn-expressing cells (arrow). Some rescue of growth is observed, which is associated with the induction of a non-contiguous patch of Ap-expressing cells that form an abnormally small D compartment. As in (B), mirr-lacZ expression is rescued at short-range in a lateral region of the wing hinge and the notum primordium is absent. A small Vn-expressing clone is also present in the extreme dorsoproximal region of the disc. (D) vn– disc containing a single large clone within prospective notal territory. Note that both the size and shape of the disc appear almost normal, as do the patterns of Ap and mirr-lacZ expression.

![Fig. 1. Vn-expressing clones in vn mutant discs.](image-url)
In the course of extending our analysis of the role of EGFR signaling in organizing both the DV and wing-notum segregations, we also generated clones of cells that express an activated form of Ras (RasV12) in vn- as well as wild-type discs. Such clones activate ap and the Iro-C genes in a strictly cell-autonomous manner (Zecca and Struhl, 2002), and hence present a novel situation in which the boundary of ap expression cannot shift away from that of Iro-C gene expression. As we describe below, the behavior of such clones suggests that during normal development, the DV boundary must shift ventrally, into a domain of relatively low EGFR/Ras activity, in order to acquire wing organizer activity.

We first examined whether the severe proliferation defect associated with vn mutant wing discs could be rescued by inducing RasV12-expressing clones during the first larval instar. In contrast to our results with Vn- and Rho-expressing clones, both of which can dramatically stimulate the growth of vn mutant discs (Fig. 1 and Fig. 2D,E), we obtained only a few vn mutant discs that showed a modest increase in size (Fig. 3A). Moreover, this increase appeared to be due to a cell-autonomous rescue of proliferation within the RasV12-expressing clone, rather than to a long-range, non-autonomous induction of growth, as observed for Vn-expressing and Rho-expressing clones (compare Fig. 3A,B; see also Fig. 1 and Fig. 2D,E). Nevertheless, such RasV12-expressing clones autonomously expressed ap, indicating that the clone border constitutes an ectopic DV compartmental interface (Fig. 3A). We therefore wondered if RasV12-expressing clones fail to rescue vn mutant discs because D cells within the clone cannot interact properly with surrounding V cells to induce organizing signals.

Given the technical difficulties of assaying RasV12 clones in vn mutant discs, we chose to test this possibility by analyzing the behavior of RasV12-expressing clones in otherwise wild-type discs.

As we observed in vn mutant discs, RasV12-expressing clones generated in wild-type, first instar discs autonomously expressed ap, but did not appear

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**Fig. 2.** Ectopic Rho expression in wild-type and vn mutant discs. (A-E) Wild-type (A-C) and vn- (D,E) discs containing clones that ectopically express Rho, monitored for the expression of Ap (B,D; red in middle panels), ap-lacZ (C; red), mirr-lacZ (B,D,E; red in middle or right panels), vgQ-lacZ (A; red), Vg (A; blue) and Wg (C; blue). The clones were induced during the first instar and are marked by absence of GFP expression (green). (A) Duplicated wing blade primordium (outlined in the right panel; the extra primordium is outlined in yellow) caused by a ventrally situated clone of Rho-expressing cells (arrowhead) which we infer has induced the formation of an ectopic D compartment. Note that expression of the vg gene (visualized by Vg protein expression, blue) is normally expressed along the DV compartment boundary (asterisks) in response to Notch signaling and in the remainder of the wing blade primordium in response to Wg emanating from the boundary. Expression of the vgQ-lacZ gene (red) depends on the vg ‘quadrant’ enhancer, which is activated by Wg signaling, but blocked by Notch signaling, thus allowing the ectopic DV boundary that forms within the duplicated wing blade primordium to be visualized (yellow asterisk). (B) Ectopic expression of mirr-lacZ and expansion of Ap expression caused by a dorsolateral clone of Rho-expressing cells. Note that ectopic mirr-lacZ is closely associated with the clone, outlined in white, in contrast to boundary of expression of Ap expression, which extends many cell diameters further ventrally. (C) Expansion of ap-lacZ expression caused by two dorsally situated clones of Rho-expressing cells located within the dorsal compartment. Wg is expressed in cells flanking the apON, apOFF interface, indicating that the boundary has organizer activity. (D) Partial rescue of growth and patterning of a vn mutant disc by clones ectopically expressing Rho. The boundaries of mirr-lacZ expression are located close to the clone, whereas those of Ap expression are located further away. (E) Extensive rescue of a vn- mutant disc by a single dorsally situated clone of Rho-expressing cells.
to stimulate growth or patterning of surrounding cells (Fig. 3C).

To assay whether the ectopic DV compartment boundaries associated with such clones have organizing activity, we examined the expression of wg (as visualized by the expression of either Wg protein or a wg-lacZ gene), which is normally induced in a thin stripe of cells flanking the DV compartment boundary by reciprocal signaling interactions between D and V compartment cells. In contrast to the normal DV boundary, we found that wg expression was not induced in ap-expressing and non-ap-expressing cells that abut along the borders of RasV12-expressing clones (Fig. 3C), although low levels of wg expression could sometimes be detected in cells scattered throughout the clone. These clones also blocked normal wg expression when they extended to the endogenous DV boundary (not shown). The results obtained with RasV12-expressing clones contrast with those obtained with Vn-expressing clones in vn mutant discs, as well as with Rho-expressing clones in either vn mutant or wild-type discs. Such Vn- or Rho-expressing clones maintained Iro-C gene expression at short range but induced the formation of larger domains of ap-expressing cells that typically extended several cell diameters further away from the clone, and hence into domains of lower EGFR signaling (Fig. 1, Fig. 2C,D). Like the normal DV compartment boundary, cells that flank the borders of these ectopic ap expression domains expressed Wg (Fig. 3B; data not shown; see also Fig. 2C). Thus, we infer that cells flanking the ectopic DV boundaries associated with RasV12-expressing clones fail to express Wg, because high levels of Ras activation in the ectopic D cells disrupt the reciprocal signaling events normally responsible for Wg induction along the DV boundary.

Wg is only one of several genes that are normally induced in cells flanking the DV boundary and are required for the organizing activity of the boundary. Another is vestigial (vg), a selector-like gene that specifies wing blade identity (Kim et al., 1996) and is upregulated both by reciprocal signaling interactions between D and V compartment cells and by Wg signaling in cells located further from the DV boundary (Williams et al., 1994; Kim et al., 1995; Kim et al., 1996; Zecca et al., 1996). We find that RasV12-expressing clones autonomously fail to express vg (as visualized by the expression of Vg protein, Fig. 3D), providing another indication that high levels of EGFR/Ras activity in D cells interfere with the reciprocal signaling interactions which normally induce the expression of 'boundary' genes, such as vg and wg along the DV compartment interface (see Discussion).

We conclude that the mere presence of an apON-apOFF interface is not sufficient to induce wg and vg expression and organize wing development. Instead, it appears that the apON-apOFF interface must be located in a region of low EGFR signaling to acquire organizer activity, a condition that is satisfied during normal development by the ventral expansion of the D compartment away from the source of high EGFR signaling. In agreement with this, we find that clones of ectopic

Fig. 3. Ras activity inhibits the organizing capacity of the DV compartment boundary. (A-E) vn- (A,B,E) and wild-type (C,D) discs containing clones ectopically expressing either RasV12 (A,C,D), Rho (B) or Ap (E) monitored for the expression of Ap (red in A,C,E), ap-lacZ (red in B,D), Wg (blue in B), wg-lacZ (blue in C) and Vg (blue in D). Clones were induced during the first instar and are marked by presence (A,C,D) or absence (B,E) of GFP expression (green). Clones in B appear in red. Overlap of red and green signals appear in yellow (B-D). (A) vn mutant disc containing a RasV12-expressing clone that induces ap expression autonomously, but fails to exert a non-autonomous effect on proliferation of neighboring vn mutant cells. The inset shows a vn mutant disc at the same magnification. (B) Rho-expressing clones (arrows) that exert a non-autonomous effect on both ap-lacZ expression and proliferation of vn mutant cells. The apON-apOFF interface is associated with Wg expression. Note that the disc is shown at 1.3x the magnification used in A. (C) Wild-type disc containing a RasV12-expressing clone that autonomously expresses Ap, but does not induce wg-lacZ expression along the clone border. (D) Clones of RasV12-expressing cells in a wild-type disc autonomously induce ap-lacZ and repress Vg expression. (E) vn mutant disc containing an Ap-expressing clone that non-autonomously rescues the proliferation of the wing blade primordium. The disc is shown at the same magnification as the disc in A.
ap-expressing cells can rescue wing growth and differentiation in vn mutant discs [Fig. 3E; similar observations have also been reported by Wang et al. (Wang et al., 2000)]. Thus, the only requirement for Vn-dependent EGFR signaling in controlling the development of the wing primordium appears to be activation of ap expression and the consequent establishment of the D compartment. Once this occurs, high levels of EGFR signaling are no longer required, but are instead detrimental, as they prevent the DV boundary from acquiring organizer activity.

DISCUSSION

Identity and source of the EGFR ligand organizing wing disc development

There are three known activating ligands for the Drosophila EGFR: Vn, Spi and Gurken (Rutledge et al., 1992; Neuman-Silberberg and Schupbach, 1993; Schnepf et al., 1996). Gurken is active only during female oogenesis (reviewed by Nilson and Schupbach, 1999) and thus is not a candidate for the EGFR ligand that organizes wing disc development. Likewise, although Spi is the primary ligand for the EGFR signaling that organizes wing disc development. However, if Spi is the primary ligand for the EGFR signaling that organizes wing disc development, then it appears to be dispensable in the wing disc (Simcox, 1997; Guichard et al., 1999; Nagaraj et al., 1999). By contrast, Vn is expressed in a dorsoproximal sector of the developing wing disc, where ap and the Iro-C genes are initially activated (Simcox et al., 1996; Wang et al., 2000). Moreover, loss of vn activity, like that of the EGFR, blocks the expression of ap and the Iro-C genes (Wang et al., 2000). These findings have led to the proposal that Vn is the EGFR ligand responsible for organizing ap and Iro-C expression and hence for controlling the growth and patterning of the wing disc (Wang et al., 2000).

Our results challenge this view. We show that localized expression of Vn in the dorsoproximal region of vn mutant discs is both necessary and sufficient to rescue normal wing development. However, we also demonstrate that ventral expression of Vn fails to rescue the development of vn mutant discs and that indiscriminate expression of Vn throughout the disc does not reorganize ap or Iro-C expression. Taken together, these results indicate that Vn performs a permissive, rather than an instructive, role in controlling ap and Iro-C gene expression. Finally, we demonstrate that ectopic dorsal expression of an active form of Spi can substitute for the absence of vn function during wing disc development. This last result confirms that Vn is normally required for the generation of an instructive EGFR ligand in the dorsal region of the disc, and poses the question of what this ligand is.

One possibility is that Vn itself is the ligand, but that it must be converted from an inert (or weakly active) precursor to an active (or more potent) form. Accordingly, the instructive cue would be a dorsally localized processing or activating factor that executes this conversion, serving a role analogous to that of Rho in activating Spi. This processing activity is unlikely to be Rho itself, because Rho does not appear to be required for either the DV or wing-notum segregations (Simcox, 1997). In principle, a Vn-converting activity could be provided by one of several additional Rho-like proteins that have recently been identified (Wasserman et al., 2000). However, Vn differs from Spi in that nascent Vn is likely to be a secreted protein, whereas nascent Spi is a transmembrane protein from which the EGF-containing extracellular domain is cleaved and released to generate the active EGFR ligand. Hence, Vn and Spi might be activated by distinct molecular mechanisms, with only Spi and structurally related Spi-like ligands requiring the action of Rho family proteins. Curiously, substitution of the single EGF repeat in Vn by that in Spi creates a potent, Rho-independent ligand for the EGFR (Schnepf et al., 1998). Hence, Vn might normally be converted to an active form by a mechanism that modifies the structure or conformation of its EGF repeat.

An alternative possibility is that the instructive EGFR signal is a presently unidentified EGFR ligand or co-factor that acts in conjunction with Vn (e.g. by forming a heterodimer with Vn or by potentiating EGFR activity by some other mechanism). Because indiscriminate expression of Vn does not ectopically activate either ap or the Iro-C genes, we would expect that such a ligand or co-factor would be locally expressed in the dorsal region of the disc. At least one additional spi-like gene has recently been identified in the Drosophila genome (Wasserman et al., 2000), providing a possible candidate for a Vn co-ligand.

We note that we obtained a single vn mutant disc in which a clone of laterally situated Vn-expressing cells appeared to act non-autonomously to activate ap expression in a nearby, more dorsal patch of cells (Fig. 1C). Although only one such exceptional disc was observed, it raises the possibility that Vn secreted by one cell can act jointly with a factor expressed by other cells to generate the instructive EGFR ligand. Such non-autonomy is consistent with a mechanism in which Vn functions in conjunction with another ligand, or in which Vn is converted from a weak to potent EGFR ligand after it is secreted.

A third possibility is that nascent Vn does not require processing to become an effective EGFR ligand, but instead that its ability to activate the EGFR is restricted to dorsoproximal regions of the wing disc by repressors generated in more ventrally situated cells. Because activated Spi induces both ap and Iro-C gene expression in the ventral region of the disc, such repressors might be selective for Vn itself, rather than being general inhibitors of EGFR activity. Alternatively, activated Spi might be a more potent activator of EGFR activity than Vn, allowing it to drive EGFR signaling in the presence of repressors that render Vn ineffective. Wg could, in principle, be such a repressor, as it is active ventrally and represses Vn-dependent EGFR signaling in early wing discs (Wang et al., 2000). However, during later development, Wg is expressed in a broad stripe in the presumptive notum that overlaps the domain of Iro-C gene expression in the prospective lateral notum. Hence, at least at this stage, Wg does not appear to block the Vn-dependent activation of the EGFR that maintains Iro-C gene expression.

EGFR signaling and the control of growth and patterning of the wing primordium

All cells within the wing imaginal disc require a minimum level of EGFR/Ras activity to sustain a normal rate of proliferation (Diaz-Benjumea and Garcia-Bellido, 1990; Diaz-Benjumea and Hafen, 1994; Prober and Edgar, 2000). It is not known whether this activity reflects the basal activity of the EGFR/Ras transduction pathway, or the response of the receptor to a specific ligand. However, it is clear that this low level of EGFR/Ras activity does not require Vn dependent EGFR signaling, as we and Wang et al. (Wang et al., 2000)
have shown that ectopic expression of Ap in vn mutant discs can rescue growth and differentiation of the wing primordium. This result demonstrates that the absence of wing development in vn mutant discs is an indirect consequence of the failure to establish an apON-apOFF interface.

During normal development, the ap and Iro-C genes are initially activated in overlapping dorsoproximal domains in response to EGFR signaling, and hence, at this early stage, it appears that most or all D compartment cells are exposed to relatively high levels of EGFR/Ras signaling (Fig. 4). Thereafter, as the wing disc grows, ventrally situated D compartment cells inherit the ‘on’ state of ap expression, even as they populate areas of the disc progressively farther from the domain of high EGFR/Ras signaling and sustained Iro-C expression. We suggest that the progressive reduction of EGFR/Ras activity in these ventrally situated D cells enables them to interact with neighboring V compartment cells to induce Wg and Vg expression and stimulate growth of the wing primordium. By contrast, early induced clones of RasV12-expressing cells autonomously express ap and experience persistent high levels of Ras activation, as indicated by sustained expression of the Iro-C genes. As a consequence, the ectopic DV boundary cannot shift outside of the domain of high EGFR/Ras signaling. We show here that cells flanking this ectopic DV boundary fail to engage in the reciprocal induction of Wg and Vg expression or to stimulate growth. Hence, the apON-apOFF interface may normally have to shift to a region of relatively low EGFR activity for the DV boundary to acquire wing organizer activity (Fig. 4).

We note that early induced clones which express EGFR\textsuperscript{L}, the constitutively active form of the EGFR, can induce the formation of ectopic D compartments that retain organizer activity (Zecca and Struhl, 2002). However, the level of constitutive EGFR/Ras activity in such EGFR\textsuperscript{L}-expressing clones appears to be significantly lower than in clones of RasV12-expressing cells (Zecca and Struhl, 2002). Consistent with this, we find that ectopic expression of EGFR\textsuperscript{L} considerably reduces but does not completely eliminate \(v_g\) expression (data not shown) (Wang et al., 2000). Hence, we infer that the levels of Ras activation in EGFR\textsuperscript{L}-expressing cells are not sufficiently high to prevent productive interactions between D and V compartment cells, thus allowing the ectopic DV compartment boundary to acquire organizer activity.

How might EGFR signaling regulate the capacity of the DV compartment boundary to function as an organizer? One possibility is that high levels of EGFR/Ras activity block the ability of cells to transduce Notch signals. During normal development, D and V cells engage in a positive auto-feedback loop of Delta/Notch and Serrate/Notch signaling that drives the reciprocal induction of Wg and Vg expression on both sides of the DV compartment boundary (Diaz-Benzumea and Cohen, 1995; Kim et al., 1995; Fleming et al., 1997; Panin et al., 1997). Hence, if high levels of EGFR/Ras activity block Notch signal transduction, then persistent high levels of Ras activity on even one side of the DV boundary would suffice to disrupt the feedback loop and block the reciprocal induction of Wg and other ‘boundary’ genes. Accordingly, the DV boundary might have to be located in a region of low EGFR activity in order to allow reciprocal Notch signaling to induce the expression of these, and perhaps other, organizer genes.

Another possibility is that the apON-apOFF interface may only be able to function as an organizer when cells on both sides are of prospective wing type. Prior to the initial activation of ap and the Iro-C genes, the nascent wing disc appears to be subdivided into mutually antagonistic domains of EGFR and Wg signaling that at least transiently define the incipient notum and wing primordia (Ng et al., 1996; Wang et al., 2000). Because ap and the Iro-C genes are initially activated in response to a common source of EGFR signaling, most or all D cells at this stage may be of notum type (Fig. 4). It is only later, when ventrally situated D cells move out of range of Vn-dependent EGFR signaling and switch to being of wing type, that inductive interactions occur across the DV boundary to create a new and stable source of Wg signaling (Fig. 4). We suggest that cells on both sides of the DV boundary may have to be of wing type for the boundary to have organizer activity. One possible reason for why this might be the case is that \(v_g\), the selector-like gene that defines the wing state, is itself an integral component of the reciprocal signaling mechanism that allows D and V cells to induce the expression of DV boundary genes (Williams et al., 1994; Kim et al., 1996; Guss et al., 2001). As we show here and in the accompanying paper (Zecca and Struhl, 2002), high levels of EGFR/Ras signaling actively maintain Iro-C gene expression (and hence the notum state) and block \(v_g\) expression (Wang et al., 2000). Hence, the DV boundary may normally have to shift ventrally, into a domain of low EGFR/Ras signaling and high Wg signaling that defines
the incipient wing state, to allow the positive feedback loop of inductive signaling to initiate across the DV compartment boundary. Once this loop is established, it would provide a stable source of Wg and other signals generated along the DV boundary that govern the subsequent growth and differentiation of the wing blade (Fig. 4).

We thank C.-M. Chen for the Tub>Gal80,y>+Gal4 transgene; E. Bier, S. Carroll, F. Casares, E. Hafen, R. Mann, A. Simcox, H. Stocker and J. Thomas for fly stocks and reagents; F. Casares, H. Stocker and A. Tomlinson for discussions; G. Campbell, F. Casares, S.-K. Chan, L. Johnston, P. A. Lawrence, R. Mann, N. Methot, H. Stocker and A. Tomlinson for comments on the manuscript; and G. Campbell for communicating results prior to publication. M. Z. is a Research Associate and G. S. is an Investigator of the Howard Hughes Medical Institute.

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