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Spitz and Wingless, emanating from distinct borders, cooperate to establish cell fate across the Engrailed domain in the *Drosophila* epidermis

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SUMMARY

A key step in development is the establishment of cell type diversity across a cellular field. Segmental patterning within the *Drosophila* embryonic epidermis is one paradigm for this process. At each parasegment boundary, cells expressing the Wnt family member Wingless confront cells expressing the homeoprotein Engrailed. The Engrailed-expressing cells normally differentiate as one of two alternative cell types. In investigating the generation of this cell type diversity among the 2-cell-wide Engrailed stripe, we previously showed that Wingless, expressed just anterior to the Engrailed cells, is essential for the specification of anterior Engrailed cell fate. In a screen for additional mutations affecting Engrailed cell fate, we identified *anterior open/yan*, a gene encoding an inhibitory ETS-domain transcription factor that is negatively regulated by the Ras1-MAP kinase signaling cascade. We find that Anterior Open must be inactivated for posterior Engrailed cells to adopt their correct fate. This is achieved by the EGF receptor (DER), which is required autonomously in the Engrailed cells to trigger the Ras1-MAP kinase pathway. Localized activation of DER is accomplished by restricted processing of the activating ligand, Spitz. Processing is confined to the cell row posterior to the Engrailed domain by the restricted expression of Rhomboid. These cells also express the inhibitory ligand Argos, which attenuates the activation of DER in cell rows more distant from the ligand source. Thus, distinct signals flank each border of the Engrailed domain, as Wingless is produced anteriorly and Spitz posteriorly. Since we also show that En cells have the capacity to respond to either Wingless or Spitz, these cells must choose their fate depending on the relative level of activation of the two pathways.

Key words: *wingless*, *anterior open*, *yan*, DER, Spitz, Rhomboid, MAP kinase, *Drosophila*

INTRODUCTION

The establishment of pattern across a cellular field requires the generation of a restricted signaling source. In the *Drosophila* embryo, cellular fields are initially established with the subdivision of the body plan into parasegments (Martinez Arias and Lawrence, 1985). Signals emanating from cells at the boundary between adjacent parasegments guide cellular patterning across each parasegment in the embryonic epidermis (Baker, 1988; Martinez Arias et al., 1988; Heemskerk and DiNardo, 1994; Bokor and DiNardo, 1996). Similar mechanisms also act in patterning the imaginal discs, as signals emanate from compartment boundaries, which are inherited from the embryonic parasegment boundaries (Basler and Struhl, 1994; Capdevila and Guerrero, 1994; Tabata and Kornberg, 1994; reviewed in Lawrence and Struhl, 1996).

In both the embryo and imaginal discs, these signals act to establish cell type diversity across the field. In the embryonic epidermis, one of these signals is Wingless (Wg), a member of the Wnt gene family (Cabrera et al., 1987; Rijsewijk et al., 1987; Baker, 1988). It is expressed just anterior to cells expressing the homeoprotein Engrailed (En; Ingham et al., 1988). We are focussing on the establishment of subfates within the En expression domain as a model for the generation of cell type diversity across an embryonic parasegment.

There are two phases to patterning in the embryonic epidermis. First, reciprocal signaling occurs between Wg- and En-expressing cells, serving to consolidate the subdivision of the body plan into parasegments. (DiNardo et al., 1988; Martinez Arias et al., 1988; Bejsovec and Martinez Arias, 1991; Heemskerk et al., 1991; Cumberledge and Krasnow, 1993; Ingham, 1993). Subsequent to this early phase of signaling, the fates of cells across the parasegment are established (Bejsovec and Martinez-Arias, 1991; Dougan and DiNardo, 1992; reviewed in DiNardo et al., 1994). In the ventral epidermis, although Wg signaling is required to establish some cell fates, the interactions that establish most

of the diversity of cell types are not fully understood (Bejsovec and Martinez-Arias, 1991; Bejsovec and Wieschaus, 1993; Lawrence et al., 1996). Previously, we showed that Wg helps to generate cell type diversity within the En domain by directing anterior En cells to adopt one of two alternate fates (Dougan and DiNardo, 1992). However, the question remained as to what specifies posterior En cell fate.

Here we show that a second signal, Spitz (Spi), establishes posterior En cell fate. We find that Spi is generated by cells just posterior to the En cells, since they express Rhomboid, which is essential for production of the secreted, active form of Spi. Spi is a ligand for the *Drosophila* Epidermal Growth Factor Receptor (DER; Rutledge et al., 1992; Schweitzer et al., 1995b). We show that DER is required cell autonomously for the establishment of posterior En cell fate. Finally, we find that both rows of En cells are competent to respond to either Wg or Spi. If DER signaling is hyperactivated in the En cells, both rows assume the denticle fate. Reciprocally, if Wg signaling is hyperactivated, both rows assume the smooth fate. Thus, in each row of cells within the En domain, the relative strengths of two opposing signals, Wg and Spi, act to establish proper subfates.

MATERIALS AND METHODS

Stocks

aop^{IP}, *aop*^{IIS}, *flb*^{3F} and *wg*^{IL} (*wg*^{ts}) were from Tübingen. Df(2L)dp-79b was from Bloomington; *argos*$^{\Delta 7}$ was from Freeman et al. (1992). Wild type and constitutively active UAS-Aop are described in Rebay and Rubin (1995); UAS-secreted Spi, UAS-Rhomboid are described in Schweitzer et al. (1995b) and Golembo et al. (1996a); UAS-Arm-S10 is described in Pai et al. (1997). UAS-Ras1$^{val\text{-}12}$ and En-GAL4 were generous gifts from C. Klämbt (Scholz et al., 1997) and A. Brand, respectively. Two criteria demonstrate that En-GAL4 expression is restricted to the En cells: the expression of *lacZ* (using a UAS-*lacZ* transgene) driven by En-GAL4 (not shown), and that the effect of activated Armadillo is restricted to the En cells (see Fig. 4), since it is known that activating the Wg signaling pathway in other cells is sufficient to change their fates (Pai et al., 1997). To construct a dominant negative version of DER, a type I DER cDNA (Schejter et al., 1986) was inserted into the pUAST vector, with a termination codon 13 amino acids C-terminal to the transmembrane domain. The protein thus produced has the capacity to form inactive dimers with the endogenous wild-type receptor (see Freeman, 1996).

Cuticle screen

We screened existing mutant collections (Nüsslein-Volhard et al., 1984; Bier et al., 1989; Karpen and Spradling, 1992) by analyzing at

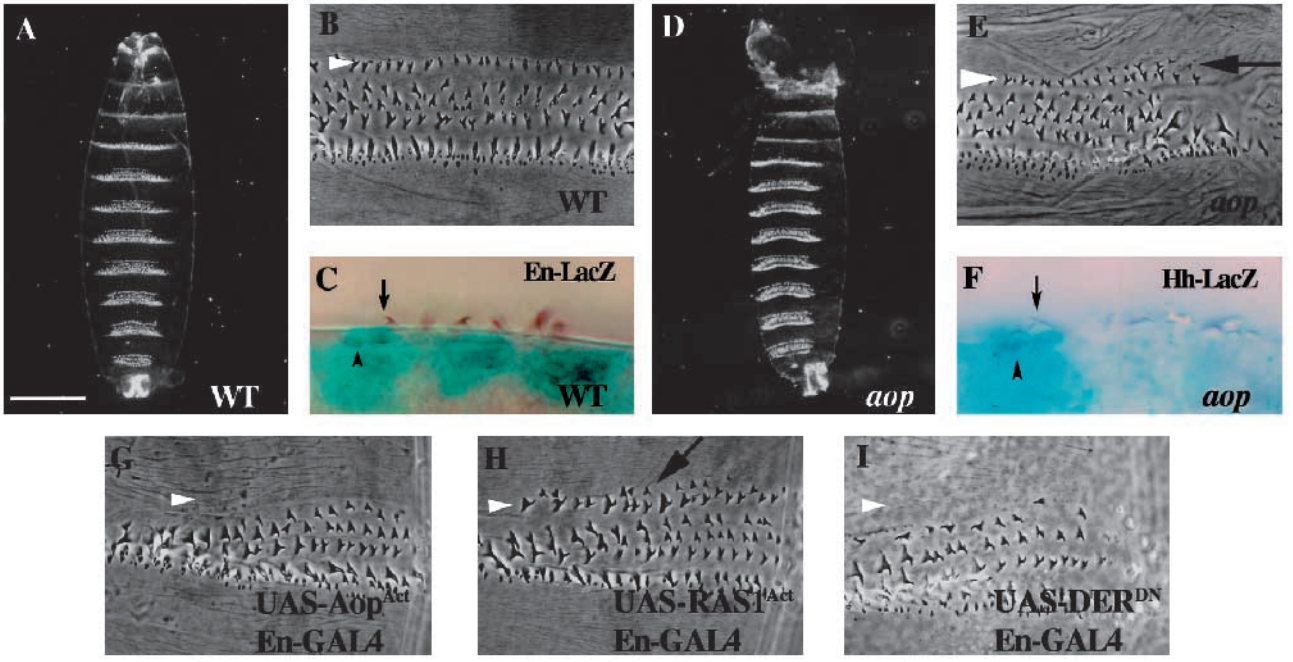

Fig. 1. Extra denticles are produced in *aop* mutants. Cuticle pattern; dark field (A,D); phase contrast (B,E,G–I); Nomarski optical sections of one denticle belt (C,F), where En-expressing cells are visualized by β-galactosidase activity produced by a *lacZ* reporter. Anterior is up in all panels except in C and F, where it is to the left. (A) Wild type. (B) Wild type; the first denticle row (white arrowhead) is small, pointing anteriorly. (C) Wild type, longitudinal optical section; thick clear horizontal line is the cuticle layer with denticles visible above and epidermal cells just below. Some denticles are slightly out of focus. Anterior En cell underlies smooth cuticle (arrowhead); posterior En cell underlies a first row denticle (arrow). Blue signal below hypodermis is irrelevant CNS expression. Note that the En stripe actually varies in width from two to three cells, with only the posteriormost cell adopting a denticle fate. Due to the variability in width, and the difficulty in capturing all cells in the same focal plane due to their hexagonal packing, throughout the text we consider it as a 2-cell-wide stripe. (D) *aop* /Df(2L)dp-79b. (E) *aop* /Df(2L)dp-79b; extra denticles anterior (arrow) to the normal first row (white arrowhead). (F) *aop*, longitudinal optical section; anterior En cell (arrowhead) forms a denticle. (G) UAS-Aop^{Act}/En-GAL4. First row denticles are missing (arrowhead), indicating that posterior En cells adopt smooth fate. (H) UAS-Ras1^{Act}/En-GAL4. Denticles (arrow) appear anterior to first row (white arrowhead), indicating that anterior En cells adopt denticle fate. (I) UAS-DER^{DN}/En-GAL4. A dominant negative form of DER cell autonomously blocks posterior En cells from adopting denticle fate (white arrowhead). Embryos carrying only UAS-DER^{DN} have no phenotype. Bar, 50 μm in A,D; 10 μm in B,C,E-I.

least 50 larval cuticles (van der Meer, 1977) from each line for ectopic denticles. Other mutants with specific pattern defects will be described elsewhere. To correlate cuticle pattern with En-expressing cells, we performed β-galactosidase activity stains (Heemskerk and DiNardo, 1994) using an En-*lacZ* reporter construct for wild type and wg^{ts} (line F; Kassis, 1990), and a *hedgehog-lacZ* reporter for *aop* (Gaul et al., 1992), since *hh* and *en* are co-expressed (Tabata et al., 1992).

Antibodies and probes

RNA in situ antibody double labeling was carried out as in Dougan and DiNardo (1992). Antisense, digoxigenin-labeled RNA probes were made from cDNAs for Wg, Hh, Rhomboid and Argos (Baker, 1987; Bier et al., 1990; Freeman et al., 1992; Lee et al., 1992). Anti-En MAb4D9 hybridoma supernatant was used undiluted (Patel et al., 1989); polyclonal anti-Wg was used 1:100 (R. Nusse); anti-β-galactosidase (Cappel) was used at 1:1,000; monoclonal anti-diphospho-ERK was used 1:1000 (Gabay et al., 1997; Yung et al., 1997; Sigma cat. #M8159); biotinylated antibodies (Vector) and horse radish peroxidase conjugated streptavidin (Chemicon) were used at 1:400; Fluorescein- and Rhodamine-labeled secondary antibodies (Jackson) were used at 1:300. Stained embryos were equilibrated in 80% glycerol and photographed as whole mounts or as fillets.

RESULTS

MAP kinase-induced inactivation of Aop determines En cell fate

The larval cuticle comprises a repeated array of precisely patterned denticle belts interspersed with smooth cuticle (Fig. 1A). In abdominal segments, each of these belts is made up of 6 rows of denticles, where each row is of a characteristic size and orientation reflecting fate decisions made by the underlying cells (Fig. 1B). Using a *lacZ* reporter gene expressed in the En cells, we demonstrated that the anterior En cells normally produce smooth cuticle (Fig. 1C, arrowhead), while the posterior En cells produce denticles and, thereby, form the first row of each belt (Fig. 1C, arrow; Hama et al., 1990; Dougan and DiNardo, 1992). Thus, cells in the En domain adopt either a smooth or denticle fate depending on their position. To identify genes involved in specifying En cell fates, we screened existing collections of mutants for those in which anterior En cells inappropriately produce denticles. We found that ectopic denticles are observed immediately anterior to the denticle belts in *aop* mutants (Fig. 1E, arrow). The extra denticles are located at the lateral edges of denticle belts, and are more commonly observed in the posterior segments (see Discussion). To determine whether the En cell fates were altered in these mutants, we visualized the En cells with a *lacZ* reporter construct. We found that the anterior En cells produce denticles instead of the normal smooth cuticle (Fig. 1F, arrowhead). Thus, *aop* function is required for some anterior En cells to adopt the smooth cell fate.

aop is allelic to *yan*, an ETS-domain containing DNA-binding protein (Rogge et al., 1995). Since *aop* was the first described mutation at this locus, we retain this designation for the gene and refer to *yan* mutants as alleles of *aop* (aop^{yan}). Aop represses the transcription of target genes during photoreceptor development and during the specification of dorsoventral cell fates in the embryonic ectoderm (Lai and Rubin, 1992; Tei et al., 1992; O'Neill et al., 1994; Rebay and Rubin, 1995; Rogge et al., 1995; Gabay et al., 1996). Repression of target genes by Aop is relieved through phosphoryla-

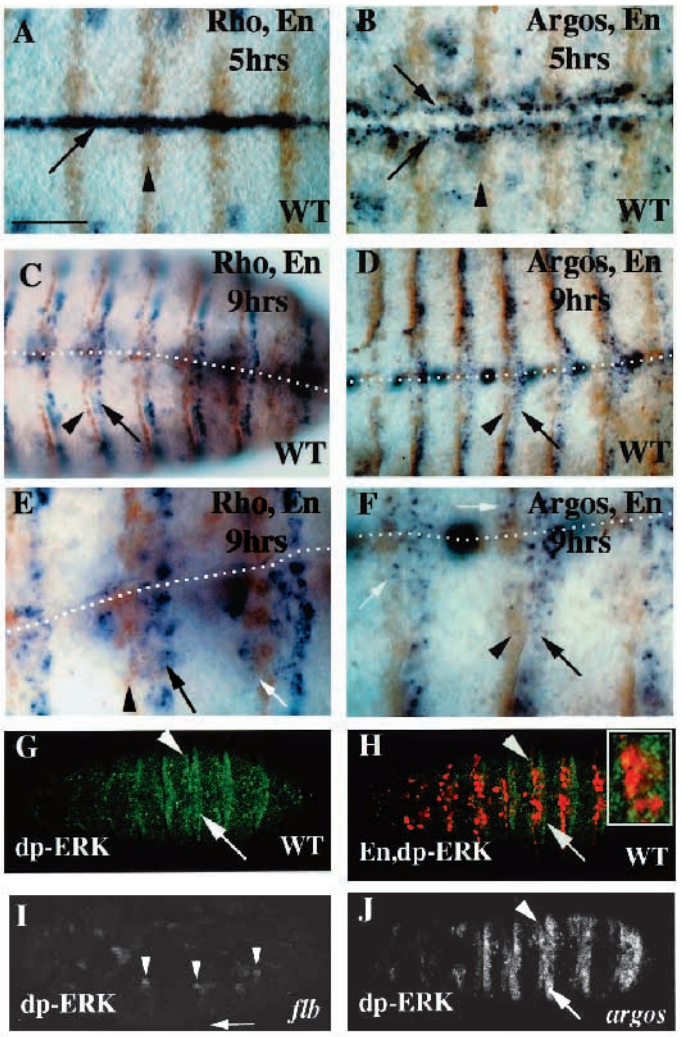

Fig. 2. A switch from midline to transverse DER signaling. (A-F) Wild type, doubly labeled for En protein (brown) and either Rhomboid RNA (A,C and E; blue), or Argos RNA (B,D and F; blue). (A) 5 hours AEL. Rhomboid is expressed in ventral midline cells (arrow), perpendicular to segmental En stripes (arrowhead). (B) 5 hours AEL. Argos is expressed in cells flanking ventral midline (arrows), perpendicular to segmental En stripes (arrowhead). (C,E) 9 hours AEL. Rhomboid expression has switched to a transverse pattern (arrow), just posterior to each En stripe (arrowhead). Some residual Rhomboid expression is observed near ventral midline (dotted line) within and anterior to En stripe. (D,F) 9 hours AEL. Argos expression has switched to a transverse pattern (arrow), just posterior to each En stripe (arrowhead). Some Argos expression is visible within En cells (white arrow), including those away from midline. (G-I) 9 hours. AEL, Confocal micrographs. (G,H) Wild type; (G) anti-dp-ERK (green); (H) anti-En (red) merged with Anti dp-ERK (green) image in G. Activated MAP kinase is present in a stripe (arrow) just posterior to the En domain (arrowhead; magnified in inset in H). Note that activated MAP kinase is visible within the En cells also. (I) flb^{3F}, lateral view (germ-band is not retracted in *flb* mutants). Transverse stripes of dp-ERK are missing (arrow), demonstrating that activated Map Kinase at the segment border depends on DER. Arrowheads point to tracheal expression of dp-ERK, which is dependent on *breathless* RTK activity. (J) $argos^{\Delta 7}$; note de-repression of dp-ERK, signifying increased intensity and broader domain of DER signaling,. Bar, 50 μm in G-I; 30 μm in C,D; 10 μm in A,B,E,F.

tion by MAP kinase, which, in turn, is under the control of Ras1 (Rebay and Rubin, 1995).

Since *aop* activity is required for anterior En cells to adopt the smooth cell fate, we tested whether Aop activity was sufficient to force posterior En cells to produce smooth cuticle instead of first row denticles. We used a constitutively active form of Aop, where all eight MAP kinase consensus phosphorylation sites were mutated and drove its expression in the En cells using the UAS/GAL4 system (Brand and Perrimon, 1993; Rebay and Rubin, 1995). While En-GAL4 embryos carrying UAS-Aop^{WT} exhibit normal denticle pattern, such embryos carrying UAS-Aop^{Act} are missing the normal first denticle row of each belt (Fig. 1G, arrowhead). Thus, if posterior En cells express a form of Aop that can not be inhibited by MAP kinase, then these cells adopt the smooth fate. This suggests that, normally, Aop must be inactivated in the posterior En cells for them to adopt denticle fates.

Given that the Ras1-MAP kinase cascade is responsible for inhibiting Aop function in other tissues, it was a good candidate for inactivating Aop in the posterior En cells. If this pathway was indeed involved, then inappropriate activation of the pathway should mimic the *aop* mutant phenotype and allow anterior En cells to incorrectly produce denticles. To test this, we examined embryos expressing a constitutively active form of Ras (UAS-Ras1$^{val\text{-}12}$) in the En cells. We found that these embryos have an ectopic row of denticles anterior to the normal first row, corresponding to the location of the anterior En cells (Fig. 1H, arrow). Thus, the anterior En cells are misspecified by ectopic Ras1-MAP kinase activity, similar to the effects of loss of *aop* function. This suggests that Ras1-MAP kinase activity may normally be responsible for inactivating Aop in the posterior En cells, allowing them to adopt the denticle fate.

En cell fate requires the *Drosophila* EGF Receptor

Since the Ras1-MAP kinase cascade is activated by receptor tyrosine kinases, we tested whether such a receptor could be involved in specifying En cell fate. For several reasons, the best candidate was the *Drosophila* EGF Receptor (DER). Firstly, in the eye, an allele of *aop^{yan}* was isolated as an enhancer of mutations in *Ellipse*, a gain-of-function allele of DER (Rogge et al., 1995). Secondly, DER is ubiquitously expressed epidermis throughout embryogenesis and is required early for ventral-to-lateral patterning, as is Aop (Raz and Shilo, 1993; Schweitzer et al., 1995b; Gabay et al., 1996; Golembo et al., 1996a). Finally, at later stages, DER is required for cells to adopt denticle fates (Clifford and Schupbach, 1992; Raz and Shilo, 1993; reviewed in Schweitzer and Shilo, 1997).

To address whether DER function was required for posterior En cells to adopt their correct fate, we expressed a dominant negative form of DER specifically in En cells. These embryos lack the first denticle row, corresponding to the position of the posterior En cells (Fig. 1I). Therefore, DER is autonomously required for the posterior En cells to adopt a denticle fate. We next determined whether there was a source of DER ligand positioned appropriately to signal to the En cells.

The Spitz source is posteriorly adjacent to En cells

It seemed likely that DER would be activated by Spi, its ligand in many other contexts. Spi is ubiquitously expressed as an inactive membrane-bound molecule with homology to TGF-α (Rutledge et al., 1992). A processing event, which requires Rhomboid (Rho) activity, releases active ligand. Thus, the spatially regulated expression of Rho marks cells that are the source for active, secreted Spi (Schweitzer et al., 1995b; Gabay et al., 1996, 1997). These cells can trigger activation of DER in adjacent cells. For example, between 3 and 5 hours after egg laying (AEL), Spi activity emanating from the ventral midline specifies ventral cell fates (Golembo et al., 1996a). At this time, a stripe of Rho is visualized along the ventral midline, perpendicular to the En stripe (Fig. 2A; Bier et al., 1990).

Later (8 to 9.5 hours AEL) a stripe of Rho RNA appears posteriorly adjacent to the En cells (Fig. 2C,E; Bier et al., 1990). En cells adopt their final fates during this period (Dougan and DiNardo, 1992). Thus, the expression of Rhomboid suggests that there is a novel source of active Spi ligand at the appropriate time and place to influence En cell fate.

To test directly whether the DER pathway was activated in these transverse stripes, we examined the spatial distribution of activated MAP kinase, using an antibody that is specific to the di-phosphorylated (active) form of MAP kinase (dp-ERK; Gabay et al., 1997; Yung et al., 1997). In late stage embryos (9.5 hours AEL), we detected a stripe of activated MAP kinase (Fig. 2G,H; arrow) just posterior to the En cells (Fig. 2H, arrowhead). This stripe is dependent on DER, since it is selectively removed in *flb* mutant embryos (Fig. 2I, arrow). In wild type, active MAP kinase is detectable within the En cells themselves, although at low levels (Fig. 2H, arrowhead and inset). Thus, it appears that DER activation indeed spreads into the En cells. We could not determine whether there is a difference between the anterior and posterior En cells.

We confirmed activation of the DER pathway by testing for the induction of a DER target gene, *argos*, the expression of which is closely correlated with regions of maximal DER activation (Gabay et al., 1996; Golembo et al., 1996b). For instance, during earlier ventral-to-lateral patterning, *argos* is expressed in the ventralmost 1- to 2-cell rows (Fig. 2B), the point of highest DER activation. However, at later stages *argos* RNA is expressed in a stripe of cells posterior to the En cells (Fig. 2D,F), coincident with the expression of Rho and the highest levels of activated MAP kinase. Taken together, these data demonstrate that a secreted DER ligand (probably secreted Spitz), produced by cells just posterior to En cells, activates DER. Furthermore, it appears that the activation of DER is graded, highest posterior to En cells and at lower levels within the En cells. This signaling corresponds to the time when fates of the En cells are being determined, which is consistent with a role for DER in determining the fates of En cells.

Anterior En cells can respond to Spitz

spitz mutants lack row one denticles (Mayer and Nüsslein-Volhard, 1988), supporting the role for Spitz in assigning posterior En cell fate. This phenotype is variable and dependent upon position within the embryo (Mayer and Nüsslein-Volhard, 1988; L. O'K. and S. D., unpublished observations; see also Szüts et al., 1997). Thus, for the remainder of the work, we consider Spitz the likely ligand, although another DER ligand may also be involved.

Since Spi instructs posterior En cells to adopt denticle fates, we wanted to test why it does not normally affect the anterior En cells, instructing them to adopt denticle fates as well. We have shown that anterior En cells can adopt denticle fates if

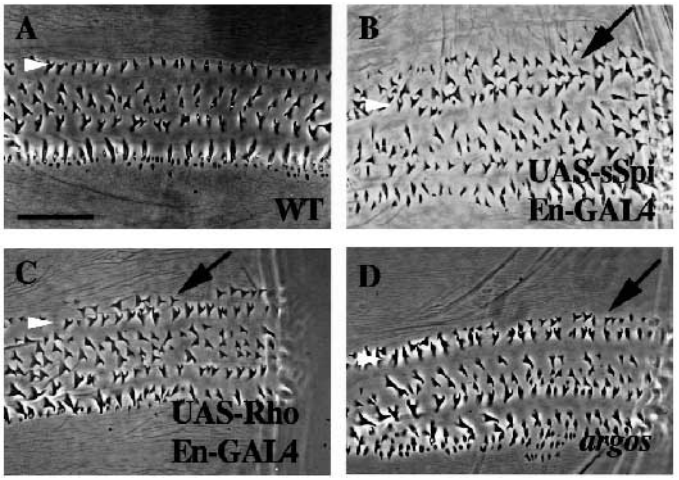

Fig. 3. Anterior En cells can respond to Spi, but are blocked from doing so by *argos*. **(**A) Wild type. (B) UAS-sSpi/En-GAL4. Denticles (arrow) appear anterior to first row (white arrowhead). These embryos have about two ectopic rows of denticles presumably because the efficient production of secreted Spi influences all En cells. (C) UAS-Rho/En-GAL4. Anterior En cells to switch to denticle fate (arrow), although less robustly than in B. (D) *argos* mutant. An extra row of denticles (arrow), made by anterior En cells, indicates that Spi reaches anterior En cells but the response is normally inhibited by *argos* activity. Bar, 10 μm in all panels.

Ras1 is activated (Fig. 1H). Thus, signal transduction components downstream of Ras1 are present in these En cells. This implies that anterior En cells either do not receive sufficient Spi to activate the pathway, or activation is blocked upstream of Ras1. To distinguish between these possibilities, we ectopically expressed the secreted form of Spi directly in En cells, exposing them to high levels of ligand. If anterior En cells were unable to respond to Spi, then they would still adopt their normal smooth fate. We found, however, that these embryos have ectopic rows of denticles anterior to the normal first row, indicating that the anterior En cells have incorrectly adopted a denticle fate (Fig. 3B, arrow). Similar results were obtained by expressing Rho in En cells (Fig. 3C, arrow). These results indicate that, if exposed to secreted Spi, anterior En cells can respond by adopting denticle fates. This implies that normally Spi does not reach anterior En cells at sufficiently high concentration to activate DER, or some factor antagonizes the Spi-DER interaction in these En cells.

A likely candidate is the secreted factor Argos, which we find is expressed just posterior to En cells, and which was shown to antagonize the response to secreted Spi in other tissues by preventing DER activation (Freeman et al., 1992; Schweitzer et al., 1995a; Golembo et al., 1996b). If this inhibitory feedback loop acts in patterning the ventral epidermis, then one would expect secreted Spi to have a broader influence if *argos* activity were removed. Indeed, in *argos* mutants anterior En cells assume a denticle fate (Fig. 3D, arrow). This suggests that, in the absence of Argos, the levels of Spi that normally reach the anterior En cells are more effective in their capacity to activate DER. Indeed, active MAP Kinase is present at apparently higher levels, and over a slightly broader domain in *argos* mutant embryos (Fig. 2J), directly demonstrating effects on the DER pathway.

Spitz and Wingless signaling have competing affects on En cell fate

Anterior En cells assume a denticle fate when *wg* function is eliminated at 8 hours AEL (Fig. 4C, arrow; (Dougan and DiNardo, 1992). Wg is expressed just anterior to the En domain, in a region of smooth cuticle (Fig. 4A, arrowhead). Thus, while Wg input instructs cells to adopt the smooth fate, activation of DER instructs cells to adopt denticle fates. The opposite response of En cells to these two signals raises the question of what fate these cells would adopt in the absence of both signals. To determine this, we blocked DER signaling by expressing Aop^{Act} in En cells while concomitantly removing *wg* function using a conditional allele. When wg^{ts} embryos carrying both En-GAL4 and UAS-Aop^{Act} were shifted to non-permissive temperature at 8 hours AEL, the En cells now adopt smooth fates (Fig. 4D, white arrowhead). This suggests that smooth cuticle is the default cell fate. Wg signaling in this context is required primarily for antagonizing DER the effect of DER signaling in anterior En cells.

The posterior En cells, which adopt a denticle fate, either cannot respond to Wg due to the absence of key signal transducers, or they do not see effective concentrations of Wg. In fact, our previous work suggested strongly that the posterior En cell did not receive Wg input (Dougan and DiNardo, 1992). We tested more precisely whether downstream signal transducers are present in posterior En cells. Cells expressing either an activated form of Armadillo or higher levels of wild-type Disheveled respond as if they have received the Wg signal (Axelrod et al., 1996; Pai et al., 1997). In embryos carrying both En-GAL4 and UAS-Arm^{S10}, the expression of activated Armadillo causes the posterior En cells to inappropriately adopt the smooth cell fate (Fig. 4E, white arrowhead). We obtained identical results expressing Disheveled (data not shown). Thus, Wg signal transducers downstream of Disheveled are present in posterior En cells. During normal patterning, these cells are probably not exposed to sufficient Wg levels to antagonize the effects of DER in these cells.

DISCUSSION

Cells within the En domain can adopt one of two alternate fates. Here we have shown that the this domain is flanked by two sources of secreted signaling molecules: Wingless at the anterior, and Spitz at the posterior. Each signal is responsible for a particular fate, and, furthermore, the En cells can respond to either signal. We conclude that proper patterning across the En domain requires a balance between these two antagonistic signals.

This novel aspect of ventral epidermal patterning makes use of an emerging strategy recently appreciated in patterning cellular fields. Distinct signals are emitted from sources established at the boundaries of the field. Since cells located between the sources can respond to either signal, their fates are influenced by proximity to the source. For instance, in the vertebrate neural tube, cells can respond to either ventralizing signals, such as Sonic Hedgehog or dorsalizing signals, such as the TGF-β family member dorsalin-1 (Basler et al., 1993; Echelard et al., 1993; Krauss et al., 1993; Riddle et al., 1993). Proximity of cells to the floor plate or roof plate, the respective signaling sources, accounts for the correct pattern of cell

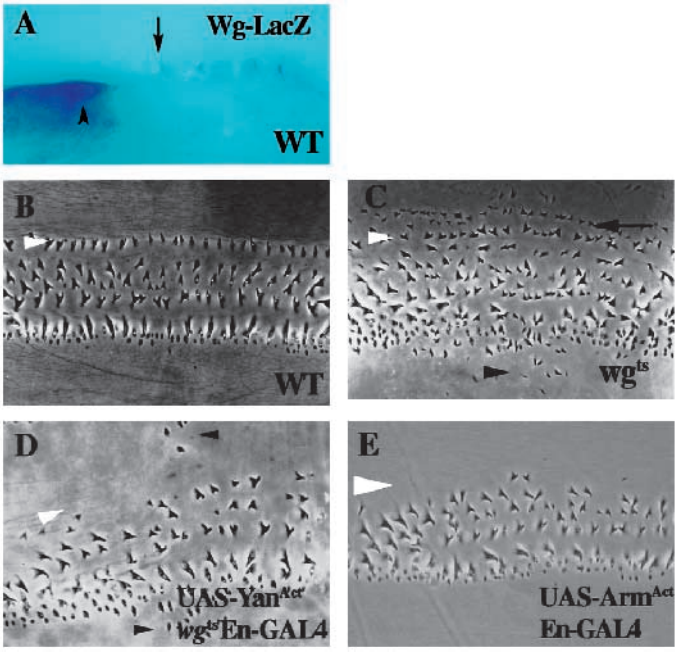

Fig. 4. Wg input also specifies En cell fate. **(**A) Wild type, longitudinal optical section. Wg expressing cells, as revealed by Wg-LacZ enhancer trap (arrowhead), are anterior to the En domain (compare with Fig. 1C). Arrow points to denticle row 1. (B) Wild type. (C) *wg*^{ts}, 8hour AEL inactivation; an extra denticle row (arrow) appears anterior to the normal first row (white arrowhead). There are also extra denticles posterior to (black arrowhead) and within belt. (D) *wg*^{ts} En-GAL4/*wg*^{ts}; UAS-Aop^{Act}/+; 8 hour AEL inactivation, as revealed by excess denticles (black arrowheads). En cells (white arrowhead) adopt smooth fate in the absence of *wg* activity if Aop cannot be inactivated. (E) UAS-Arm^{Act}/En-GAL4. Activating the Wg signal transduction pathway by expression of constitutively active Armadillo (Arm-S10) in En cells causes the posterior En cells to adopt smooth cell fate (white arrowhead). Bar, 10 μm in all panels.

fate. Similarly, during patterning of the fly leg, cells can respond to either Wg or Decapentaplegic, signals governing ventral and dorsal fates, respectively (Struhl and Basler, 1993; Basler and Struhl, 1994; Diaz-Benjumea and Cohen, 1994; Wilder and Perrimon, 1995). In this particular case, to ensure that cells will largely come under the influence of only one signal, Wg and Decapentaplegic antagonize the expression of each other (Brook and Cohen, 1996; Jiang and Struhl, 1996; Theisen et al., 1996).

Patterning requires the establishment of distinct flanking signals

The En cells can respond to transduction of either the Wg or Spi signals. This suggests that control over pattern within the En domain must be exercised by controlling ligand expression or processing of these ligands. Indeed, two successive regulatory circuits act to ensure that Wg is expressed anterior to the En domain. While the pair-rule gene hierarchy acts early to establish Wg and En expression, this pattern is consolidated by later cross-regulatory interactions between the En and Wg expressing cells (DiNardo and O'Farrell, 1987; DiNardo et al., 1988; Ingham et al., 1988; Martinez Arias et al., 1988; Bejsovec and Martinez-Arias, 1991; Heemskerk et al., 1991; Ingham et al., 1991). Only after the action of these two regulatory circuits does Wg act to specify En cell fate (Bejsovec and Martinez-Arias, 1991; Dougan and DiNardo, 1992).

Less is known of the regulatory circuits that control the expression of Rho, thus generating a source of secreted Spi. However, the control over Spi production is likely to be as elaborate as that for Wg, since there is a switch in the axis being patterned. Early, DER signaling is required for dorsoventral patterning in the epidermis, with the ventral midline as the source of secreted Spi (Raz and Shilo, 1993; Schweitzer et al., 1995b; Gabay et al., 1996; Golembo et al., 1996a). Just a few hours later, DER signaling is required for anteroposterior pattern, with cells just posterior to the En domain as the source of secreted Spi. One obvious possibility for the regulation of late Rho expression is that Hedgehog (Hh), which is produced by En cells, signals posteriorly to induce Rho. However, late Rhomboid expression was not affected by removing late *hh* function (S. D., unpublished results). We do find that boosting the level of Hh production can expand the domain of Rhomboid expression (data not shown). Thus, in late embryos Hh can modulate, but does not initiate Rhomboid expression. This suggests that the expression of Rhomboid, like Wg, will be controlled by multiple regulatory circuits.

Control of signal spread

Recent analyses have suggested that both the Wg and Spi ligands can act over some distance (Freeman, 1996; Gabay et al., 1996; Lawrence et al., 1996; Zecca et al., 1996; Neumann and Cohen, 1997). Thus, since all En cells can respond to either signal, the proper allocation of En cell fate may require restricted spread of these signals across the En domain. In considering the role of Spi, we find that expression of the DER target gene Argos is largely confined to cells posterior to the En domain, confirming that DER activation is highest at the source of Spi. However, we find that there is also activation in the En cells, although at lower levels, as revealed by following the active state of the DER signaling pathway with dp-ERK antibodies. This is consistent, in fact, with finding low levels of Argos gene expression in En cells. It is likely that the pathway is more active in posterior compared with anterior En cells, although we did not have enough sensitivity with the dp-ERK antibodies to judge this directly. Whether or not there is a differential in DER activation, it appears that low levels of secreted Spi normally reach the anterior En cell. Support for this comes from removing *argos* activity, which allows sensitive detection of which cells are exposed to Spi. Under this condition, anterior En cells adopt a denticle fate. Thus, we conclude that Spi spreads across the En domain, but its inhibitor controls signaling efficacy such that only posterior En cells normally respond (Fig. 5). This is strongly supported by the change in dp-ERK level observed in *argos* mutants.

Since we find that posterior En cells can respond to Wg signaling, the posterior En cells must normally not encounter sufficient Wg to adopt smooth cell fate (Dougan and DiNardo, 1992). This is consistent with the distribution of Wg antigen, which is skewed away from the En cells in late embryos (van den Heuvel et al., 1989; Gonzalez et al., 1991), and suggests that some mechanism exists for regulating the diffusion, accumulation or transport of the Wg ligand. Interestingly, there is evidence that transcytosis may facilitate the spread of Wg (Bejsovec and Wieschaus, 1995). Perhaps transcytosis is

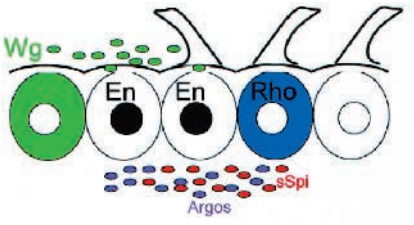

Fig. 5. Wingless and Spitz cooperate to specify Engrailed cell fate. The En-expressing cells are flanked anteriorly by a cell row producing Wg (green) and posteriorly by a cell row expressing Rhomboid (blue), which produces secreted Spitz (red). The En cell nearest the Spi source receives a higher concentration of Spi, and, thus activates the DER pathway sufficiently to specify a denticle fate. Reciprocally, En cell nearest the Wg source receives a higher concentration of Wg and adopts a smooth fate. Spi also activates the DER pathway in the Rho-expressing cell, which therefore produces and secretes Argos (purple). Argos can inhibit Spi activation of the DER pathway at a distance. This is depicted as Argos diffusing farther than Spitz, although other mechanisms are possible. As a consequence, the DER pathway is not sufficiently activated in the anterior En cell to compete out Wg signaling in this cell, and it adopts a smooth fate. Ligand distribution away from the En domain is omitted for clarity (see also Szüts et al., 1997).

spatially regulated in late embryos to restrict Wg from reaching posterior En cells. Note that the visualization of Wg ligand is not sensitive enough to reflect the domain over which it acts (Nellen et al., 1996; Neumann and Cohen, 1997). Thus, it is also possible that posterior En cells are exposed to Wg, but that active DER signaling in posterior En cells blocks the response to Wg. Such an antagonistic interaction between the effects of the Wg and DER pathways, superimposed on the graded distribution of the activating ligands, will ensure the proper order of cell fates across the En domain (Fig. 5). The *argos* mutant phenotype supports this model. Although we observe no loss of Wg expression in *argos* mutants, anterior En cells switch to denticle fate. Thus, the boost in DER activation must be competing out the effect of Wg signal transduction in the anterior En cells. The mechanism by which the Wg and Spi ligands compete could be through cross inhibition at the level of one of their respective signal transducers, or competition farther downstream, perhaps at or after target gene induction.

Competition between these two pathways also explains specific patterning errors that are observed in wild-type embryos. Occasionally, anterior En cells at the ventral midline adopt a denticle fate. This correlates nicely with some residual Rho expression we see in late embryos near the ventral midline, just anterior to the En cells (Fig. 2E). As a consequence these cells probably produce some secreted Spi. This in turn activates the DER pathway, which competes with Wg signaling, thereby occasionally causing En cells to adopt a denticle fate.

The specification of Engrailed cell fate and the DER pathway

We do not know how En cells respond to DER activation to differentiate cuticle with a denticle. Since activation of the DER pathway leads to transcriptional responses, and since we have shown that DER is required autonomously in En cells, we surmise that an undefined target gene(s) causes this response. The involvement of transcriptional regulation is supported by our observation that activation of the pathway represses, at least in some cells, the inhibitory ETS domain protein, Aop. Reciprocally, the induction of smooth cell fate by Wg signaling also appears to require transcriptional induction, as mutations in the HMG-box protein DTcf-1/Pangolin, recently implicated in Wg signaling, affect En cell fate (Brunner et al., 1997; van de Wetering et al., 1997).

Although most elements of the conserved DER signaling cassette are involved in En cell fate specification, we have noted some differences involving the ETS-domain transcription factors. First, in all reported cases, the proteins Pointed and Aop work as an antagonistic pair, competing for binding to target genes. In cells induced by DER, MAP kinase phosphorylation inhibits Aop, allowing Pointed to act positively in transcription of target genes (O'Neill et al., 1994; Rebay and Rubin, 1995; Gabay et al., 1996). This seems not to be the case here, since Pointed is expressed anterior to the En domain, rather than in the En cells in which Aop is inactivated by DER signaling (data not shown). In addition, ectopic expression of Pointed P1 did not activate the pathway in the En cells, but rather induced the same phenotype as activated Aop (data not shown). Thus, in the En cells, Aop may act by competing for binding with a novel unknown positively acting ETS protein. Alternatively, Aop could bind to sites that partially overlap with another family of transcriptional activators (Riesgo-Escovar and Hafen, 1997). A second difference is revealed by the fact that the *aop* mutant phenotype is restricted to lateralmost En cells, yet expression of an activated form of Aop can affect the fate of all En cells. This suggests that another negatively acting ETS-domain protein is expressed in the ventral epidermis. The identification of Wg and DER pathway target genes that regulate En cell differentiation should address these questions.

Several discrete temporal requirements for DER in patterning the ventral epidermis are revealed by temperature shifts using the DER flb^{ts} allele, including dorsoventral, CNS patterning and proper expression of the *gooseberry* and *patched* genes (Clifford and Schupbach, 1992; Raz and Shilo, 1992, 1993; E. R. and B.-Z. S., unpublished observations). In addition, although we have focussed only on En cell fate, temperature shifts show that formation of all denticle cell types requires activation of DER between 5 and 7 hours AEL (Clifford and Schupbach, 1992; Raz and Shilo, 1992). It is at the end of this requirement that DER activity is necessary for En cell fate, as stripes of Rho expression first appear at about 8 hours AEL. In addition, enhanced levels of DER signaling correlate with the specification of the middle rows within each denticle belt (S.D., B.-Z. S. and L.O'K., unpublished observations). Contemporaneously, Szüts et al have elegantly shown that DER activity is responsible for the anterior four rows within each abdominal denticle belt (Szüts et al., 1997).

The requirement for DER activity for several denticle cell types suggests that signaling is permissive, telling a cell to make a denticle, rather than instructive, telling a cell to make a particular denticle type. If this is true, then denticle type is conveyed by other characteristics of that cell. In the case of En cell fate, we suggest that this is due to the transcription factor En. This situation is reminiscent of the repeated use of DER during successive recruitment of different photoreceptor cells in eye patterning (Freeman, 1996). The distinct outcome at each stage is determined by the biological context of the cells

in which DER signaling takes place, as well as the other signaling pathways that synergize with or antagonize DER activity.

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