An Autoregulatory Cascade of EGF Receptor Signaling Patterns the Drosophila Egg

Jonathan D. Wasserman and Matthew Freeman*
MRC Laboratory of Molecular Biology
Cambridge CB2 2QH
England

Abstract

Intercellular signaling through the EGF receptor (EGFR) patterns the Drosophila egg. The TGFα-like ligand Gurken signals from the oocyte to the receptor in the overlying somatic follicle cells. We show that in the dorsal follicle cells this initial paracrine signaling event triggers an autocrine amplification by two other EGF ligands, Spitz and Vein. Spitz only becomes an effective ligand in the presence of the multitransmembrane domain protein Rhomboid. Consequent high-level EGFR activation leads to localized expression of the diffusible inhibitor Argos, which alters the profile of signaling. This sequential activation, amplification, and local inhibition of the EGFR forms an autoregulatory cascade that leads to the splitting of an initial single peak of signaling into two, thereby patterning the egg.

Introduction

Differentiation and pattern formation in multicellular organisms are often triggered by inductive signaling between cells. A major unresolved question is how signaling can specify patterns during development. In this paper we address how controls of EGF receptor (EGFR) signaling are integrated and the regulatory logic that allows signals themselves to elaborate complex patterns from simple origins. The Drosophila EGFR receptor mediates two inductive events that establish both body axes during oogenesis (Schüpbach, 1987; Price et al., 1989; Gonzalez-Reyes et al., 1995; Roth et al., 1995). The receptor is expressed in the somatic follicle cells, which form a columnar epithelium overlying the oocyte, whereas its ligand, Gurken, is expressed on the surface of the oocyte (Neuman-Silberberg and Schüpbach, 1996). Gurken is only expressed adjacent to the nucleus, which migrates from its initial position at the posterior of the oocyte to the dorsal-anterior. Localized Gurken thus activates the receptor first in the posterior follicle cells (stages 5–6) and then in the dorsal-anterior follicle cells (stages 8–9), establishing both the main axes of the egg. Later in development, these axes are relayed to the embryo (reviewed in Morisato and Anderson, 1995).

Not only does EGFR signaling in oogenesis specify the body axes, it also patterns the egg (for review see Ray and Schüpbach, 1996). There are two dorsal-anterior respiratory appendages, one on each side of the midline. Their differentiation and position are controlled by the EGFR: they are absent when signaling is blocked and can be repositioned by ectopic receptor activation.

Although various models have been proposed (Brand and Perrimon, 1994; Neuman-Silberberg and Schüpbach, 1994; Morimoto et al., 1996; Deng and Bownes, 1997; Queenan et al., 1997), it is not understood how dorsal signaling by Gurken and the EGFR specifies the lateral positions of the appendages. Like its mammalian homologs, the Drosophila EGFR receptor is activated by a number of ligands (reviewed in Schweitzer and Shilo, 1997; Wasserman and Freeman, 1997). Spitz and Gurken are similar to transforming growth factor α (TGFα), and Vein resembles the neu-regulins (Rutledge et al., 1992; Neuman-Silberberg and Schüpbach, 1993; Schnepp et al., 1996). Spitz is responsible for most signaling in the developing fly, while Gurken’s function is limited to oogenesis. The exact role of Vein is still unknown, although it is clearly an activating EGF ligand in some tissues (Schnepp et al., 1996; Yarnitzky et al., 1997; Szüts and Bienz, 1998). In addition to the receptor and ligands, another key component of Drosophila EGFR signaling is Rhomboid, a protein with multiple transmembrane domains (Bier et al., 1990; Ruohola-Baker et al., 1993; Sturtevant et al., 1993; Freeman, 1994). There is considerable evidence that Rhomboid regulates processing of Spitz in some tissues, producing the cleaved, active form of the ligand (Schweitzer et al., 1995b; Golembo et al., 1996a; zur Lage et al., 1997).

In the egg chamber, however, Rhomboid’s function is unclear, since its location appears to contradict this presumptive function. Contrary to other tissues, it is expressed and required in the cells that receive the inductive signal (the follicle cells), but not in the oocyte, which is the source of the ligand (Ruohola-Baker et al., 1993). This has led to the alternative proposal that Rhomboid activates EGFR signaling by enhancing reception of the signal, instead of by activating the ligand (Ruohola-Baker et al., 1993; Sturtevant et al., 1993; Schüpbach and Roth, 1994).

An explanation for the observed discrepancy in Rhomboid function is now suggested by our finding that Spitz is required in the follicle cells for normal patterning of the egg. Spitz acts as an autocrine amplifier of the initial paracrine Gurken signal and appears to be regulated by Rhomboid. We also show that the EGFR inhibitor Argos splits the EGFR activation profile into two laterally displaced peaks, thereby localizing dorsal appendage formation. This evidence suggests a revised model of dorsal specification and patterning in which the EGFR is sequentially activated by Gurken, amplified by Spitz, and inhibited by Argos. The interplay between these ligands and the receptor describes an autoregulatory circuit that accounts for the patterning functions of the EGFR. It illustrates how intrinsic regulatory properties of EGFR signaling can specify complex patterns in development.

Results

Spitz Is Required in Follicle Cells

Although Gurken is the only ligand previously reported to activate the EGFR during oogenesis, the requirement
Table 1. Germline Clones

<table>
<thead>
<tr>
<th>Genotype</th>
<th>% Fertilized</th>
<th>% Viable</th>
<th>% Zygotic Rescue</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>spitzA14</td>
<td>90.0</td>
<td>54.5</td>
<td>100</td>
<td>500</td>
</tr>
<tr>
<td>spitzSC1</td>
<td>89.4</td>
<td>49.4</td>
<td>100</td>
<td>500</td>
</tr>
<tr>
<td>argosC1</td>
<td>85.0</td>
<td>47.1</td>
<td>100</td>
<td>290</td>
</tr>
<tr>
<td>Egfr1K35</td>
<td>68.7</td>
<td>44.8</td>
<td>100</td>
<td>430</td>
</tr>
</tbody>
</table>

For each genotype, females with germline clones (see Experimental Procedures) were mated to balanced males with the same mutation. Because the dominant female sterile mutation ovoD1 was used, all the eggs laid were derived from mutant female germ cells. None of the eggs had visible phenotypes attributable to the germline clones. The eggs were collected, and the proportion fertilized is shown. Mendelian inheritance predicts that 50% of the fertilized eggs would receive the balancer (wild-type) chromosome from the father, and if the eggs were fully viable, these embryos should survive. For each genotype the viability is close to the predicted 50%. In all cases, 100% of the surviving embryos indeed had the marked balancer chromosome and not the mutant one. These results indicate that there is no requirement for Spitz, Argos, or the EGFR in the oocyte itself.

quantified this phenotype by measuring the gap between the dorsal appendages and found that their mean separation was $50 \pm 5 \mu m$ ($n = 68$) in spitz hypomorphs, compared to $73 \pm 6 \mu m$ ($n = 58$) in eggs from wild-type mothers; furthermore, in 20% of the mutant eggs the dorsal appendages were fused at the point of attachment, a phenotype never seen in wild-type eggs.

Spitz could be required in the oocyte or in the somatic follicle cells that surround it. To examine this, germline clones of spitz null mutations were generated; in these, the oocyte is mutant but the follicle cells are wild type. Contrary to an earlier report (Mayer and Nüsslein-Volhard, 1988) this causes no defects, either in patterning the egg or in the viability or patterning of the embryos derived from such eggs (Table 1). We have also confirmed that there is no requirement for the EGFR in the oocyte, only in the follicle cells (Table 1). There is therefore an essential function for the EGFR ligand Spitz in dorsal-anterior patterning of the egg, and it is required only in the somatic follicle cells, where the receptor is also needed.

This was further tested by using Flp/FRT-mediated mitotic recombination (Xu and Rubin, 1993) to induce unmarked Minute spitz null clones in the follicle cells. These clones confirm that Spitz is needed in the follicle cells. In collections of eggs from females in which clones had been induced, 21.8% of eggs ($n = 385$) had a loss of the gap between the two dorsal appendages, as was seen in the spitz hypomorphs. The phenotypes ranged from partially fused (forked) appendages (5.2%) to complete fusion (16.6%) (Figure 1E). No eggs from control females ($n = 134$) had this class of phenotype (see Experimental Procedures for details of controls). These clones were induced by expressing the flipase under the control of the hsp70 promoter. As the clones were unmarked, their size and precise location were not scorable. Clones were also induced exclusively in the follicle cells using a tissue-specific enhancer (Duffy et al., 1998). These also produced the same fused appendage phenotypes (not shown). The phenotype caused by loss of
Spitz resembles that caused by reduction of EGFR signaling (Figure 1F; Schüpbach, 1987). In contrast, when EGFR signaling is removed altogether in a gurken mutation, there is a complete loss of dorsal appendage and no indication of dorsoventral polarity (Figure 1G; Schüpbach, 1987). The difference between loss of Gurken and Spitz implies that in the absence of prior Gurken signaling from the oocyte, no Spitz signaling within the follicle cells occurs; in contrast, Gurken signaling is unaffected by loss of Spitz.

In other tissues Rhomboid appears to activate Spitz/EGFR signaling (reviewed in Wasserman and Freeman, 1997) leading us to suspect that Rhomboid mediates autocrine Spitz signaling in the follicle cells. Consistent with this idea, the phenotype caused by loss of Spitz from the follicle cells is similar to that caused by loss of Rhomboid. Expression of antisense rhomboid causes loss of dorsal tissue and fusion of the appendages in eggs from heat-shocked females expressing HS-as-rho (Figure 1H; for details see Ruohola-Baker et al., 1993). Unmarked follicle cell clones of a rhomboid null mutation also give fused appendage phenotypes; as with spitz clones, these range from mild to severe fusions (not shown). Like Spitz and the EGFR, Rhomboid is not needed in the oocyte (Mayer and Nüsslein-Volhard, 1988; Ruohola-Baker et al., 1993), implying that it, too, is only required in the follicle cells.

**Figure 2. Follicle Cell Expression of spitz, rhomboid, and vein**

(A–D) A single ovariole comprises a string of egg chambers of increasing maturity (A). In situ hybridization shows that spitz is expressed in all follicle cells from region 2 of the germarium (g), detail in (C), through stage 13 (B). A stage 10a egg chamber shows that the staining is confined to the follicle cell layer (fc) and is absent from the oocyte (D) is detail of the region boxed in [B]. Note that the intense staining seen in the nurse cells (e.g., arrowhead in [B]) is background that is seen with many probes; unlike the rest of the signal it is present in control hybridizations with a sense strand probe, and it is seen even in spitz germline clones.

(E) At stage 10b/11 rhomboid is expressed in two stripes (shown by arrows), one on each side of the midline.

(F) vein is expressed in similar stripes of cells at stage 10b.

(G) vein is also expressed in the follicle cells around the micropyle and in a posterior group of follicle cells at stage 12 (see arrow in [H]).

(H) vein is ectopically expressed in eggs from fs(1)K10 mothers in which Gurken is mislocalized circumferentially around the anterior of the oocyte and there is consequent ectopic activation of the EGFR (Neuman-Silberberg and Schüpbach, 1993). Vene expression mirrors this ectopic activation; the posterior expression of vein is unaffected. All staging according to Spradling (1993).

Vein May Also Contribute to Dorsal-Anterior Patterning

The expression of the neuregulin-like EGFR ligand vein was also examined (Figures 2F–2H). We find that it is also expressed in two stripes of follicle cells at stage 10b (Figure 2F). Interestingly, vein expression is dependent on EGFR signaling: it is ectopically expressed in fs(1)K10 eggs (Figure 2H) and absent from gurken null eggs (not shown), establishing another potentially important feedback mechanism. This suggests that the autocrine amplification of EGFR signaling also involves Vein, although in this case the feedback occurs by direct transcriptional activation of the ligand. vein expression

spitz Is Expressed in Follicle Cells

spitz is expressed uniformly in all follicle cells from very early in oogenesis (region 2 of the germarium) through stage 13, when egg patterning is complete (Figures 2A–2D). No transcript can be detected in the oocyte, consistent with its lack of Spitz requirement. This expression domain coincides exactly with that of the EGFR itself (Sapir et al., 1998). spitz expression in follicle cells is unaffected by EGFR signaling (not shown); in egg chambers from gurken or EGFR mutant mothers the spitz expression is unaltered. The same is true of egg chambers from fs(1)K10 mothers, which have ectopic EGFR signaling (Wieschaus et al., 1978; Neuman-Silberberg and Schüpbach, 1993). These expression data show that Gurken/EGFR signaling does not affect Spitz transcription, implying that the dependence of Spitz signaling on prior Gurken signaling must be posttranscriptional.

In contrast, rhomboid is expressed in a dynamic pattern in follicle cells and is dependent on EGFR signaling (Ruohola-Baker et al., 1993). At stages 9–10a of oogenesis, it is expressed in a central patch of the dorsal-anterior follicle cells, and this resolves to a stripe of cells on either side of the dorsal midline by stage 10b (Figure 2E; Ruohola-Baker et al., 1993). In the absence of EGFR signaling, rhomboid expression is lost and, conversely, it is ectopically expressed in fs(1)K10 egg chambers (Ruohola-Baker et al., 1993). These expression profiles of spitz and rhomboid are consistent with Gurken signaling from the oocyte activating the expression of rhomboid in the follicle cells. This may in turn allow Spitz to become an autocrine ligand in the follicle cells and thus establish an autocrine amplification of the initial paracrine signal.
has also been found to be dependent on EGFR signaling during embryogenesis (T. Volk, personal communication).

Genetic Interactions between spitz, rhomboid, vein, and ras1
Strong synergistic genetic interactions between spitz and rhomboid support the idea that they act in the same pathway to pattern the egg. First, the phenotype caused by reducing rhomboid is made more severe by reducing spitz. HS-as-rho flies lay a small proportion of eggs (0.4%, n = 1002) with forked appendages even without heat shock (see also Ruohola-Baker et al., 1993). When the dose of spitz is halved, the proportion of eggs with a ventralized phenotype increases to 8.8% (n = 1253). Second, the phenotype caused by overexpressing rhomboid is suppressed by halving spitz. Ectopic expression of rhomboid in line HS-rho85b (see Experimental Procedures) causes an increased gap between the dorsal appendages and moves them forward—a typical “dorsalized” phenotype (Noll et al., 1994). When the dose of spitz is halved this phenotype is strongly suppressed (Figures 3A–3E). Since spitz heterozygotes have no phenotype in a wild-type background, these interactions suggest that Rhomboid acts in the same pathway as Spitz in the follicle cells (although it does not rule out the possibility that they work in parallel unrelated pathways).

Strong genetic interactions between spitz and ras1 mutations were also observed, consistent with Spitz activating the EGFR and subsequently the Ras1/MAP kinase pathway. ras1ΔΔΔ is a viable, hypomorphic allele of ras1 (Schmon and Berg, 1996). A small proportion of eggs from ras1ΔΔΔ homozygous females have fused dorsal appendages, but this phenotype is dramatically enhanced by halving the spitz dose (Figures 3F–3J). In the same genetic test, vein mutations also enhanced the ras1ΔΔΔ phenotype (Figure 3K), implying that Vein also plays some part in the autocrine amplification of EGFR signaling.

The results described thus far imply that oocyte-derived Gurken cannot sufficiently activate the EGFR receptor in the dorsal anterior follicle cells; a secondary autocrine amplification of signaling is required, using another TGF-β-like ligand, Spitz, and also the neuregulin-like Vein. Our results also suggest a model for how this is regulated. Rhomboid is expressed in the follicle cells in response to initial Gurken signaling; the combination of Rhomboid, Spitz, and the EGFR in the follicle cells then allows Spitz to become an effective autocrine ligand (by mechanisms still unclear). In contrast, vein transcription is directly controlled by EGFR signaling.

argos is expressed in Response to Amplified EGFR Receptor Signaling
The expression of the secreted EGFR inhibitor, Argos (Freeman et al., 1992; Schweitzer et al., 1995a), is dependent on EGFR signaling in many tissues (Golembo et al., 1996b). Consistent with this, argos is expressed in the dorsal-anterior follicle cells at the time when EGFR signaling occurs (Figures 4A and 4B; Queenan et al., 1997). At stage 11 the RNA is detectable in a single, T-shaped group of cells centered on the dorsal midline, and by stage 13 it, like rhomboid and vein, is found in two groups of cells, one on each side of the midline. As elsewhere, argos expression is dependent on EGFR activation: in gurken mutant egg chambers it is lost, and it is ectopically expressed in fs(1)K10 egg chambers (not shown).

Is argos expression dependent on Spitz amplification of EGFR signaling? To test this, we examined whether
Spitz contributes to a signaling threshold required to induce argos expression. argos expression is normal in eggs from mothers with reduced Ras1 (ras1<sup>112a</sup>/ras1<sup>112a</sup>, see above), but when Spitz is halved (spi<sup>1140</sup>/;ras1<sup>112a</sup>/ras1<sup>112a</sup>) dorsal-anterior argos expression is abolished in most egg chambers (Figures 4C and 4D). Therefore, there is indeed a threshold of EGFR signaling required to switch on argos, and both Gurken and Spitz participate in reaching this threshold.

The need for amplification of EGFR signaling mediated by Spitz/Rhomboid to induce argos expression was also confirmed by examining eggs expressing antisense rhomboid. Dorsal-anterior argos expression in these eggs is significantly reduced (Figures 4E and 4F); it is often completely lost (although low-level staining can be seen in some egg chambers).

Argos Is Required to Position EGFR Activation
The initial expression of argos at the dorsal midline led us to speculate that it might cause a reduction of EGFR signaling near the midline, thereby splitting the single signaling peak into two. The resulting twin peaks of EGFR activation would then specify the location of the dorsal appendages (see Figure 7). A prediction of this model is that loss of Argos should remove inhibition of the EGFR at the midline and produce a single peak of signaling, leading to the formation of a fused appendage phenotype. The eggs from females with hypomorphic argos mutations (argos<sup>117</sup>/argos<sup>W11</sup>) were examined (Figure 5). A significant proportion of these eggs (13.8%, n = 500) have a partially or, in the most severe cases, fully fused phenotype. The same fused appendage phenotype was observed in follicle cell clones of an argos null mutation (argos<sup>117</sup>). Unmarked clones were induced, like those for spitz mutants described above. A proportion (17%, n = 182) of eggs from females in which clones were induced had appendage fusions, compared to none (n = 601) from control females (see Experimental Procedures). Conversely, complete loss of Argos from the oocyte in germline mosaics affects neither egg patterning nor later embryonic development (Table 1). These data imply that there is a requirement for Argos in eggshell patterning and that, as with Spitz, Rhomboid, and the EGFR, this requirement is confined to the follicle cells.

Direct Evidence for Twin Peaks of EGFR Activation
We propose that Argos modifies the initial EGFR activation profile in the follicle cells, producing twin peaks of activity displaced from the midline (Figure 7). These specify the position of the dorsal appendages. Direct evidence for a transition from one to two peaks of signaling was obtained with an antibody that recognises only the activated, diphosphorylated form of MAP kinase, a key member of the signal transduction pathway downstream of the receptor. At stages 9-10, there is a single domain of activated MAP kinase in the follicle cells,
We have examined how the activation of the EGF receptor triggers dorsal-anterior follicle cell fate and establishes the location of the dorsal appendages. The data presented here reveal that there are three distinct stages of dorsal determination: the sequential initiation, amplification, and repositioning of EGFR signaling (Figure 7). Two key transitions occur during this process, both of which are dependent on EGFR activation itself and are therefore autoregulatory. First, there is a paracrine to autocrine shift in EGFR signaling: what starts as a signal between cell layers, from the oocyte to the somatic follicle cells, is amplified and prolonged by signals emanating from the follicle cells themselves. Second, the consequent inhibition of EGFR signaling by Argos alters the profile of activation and therefore positions the dorsal appendages. Together, these mechanisms comprise an autoregulatory network that controls inductive patterning at the anterior of the egg.

A Shift from Paracrine to Autocrine Signaling

Gurken is the only EGFR ligand known to be required in the oocyte itself. Its expression is limited, initially to the posterior region, where it acts to specify posterior follicle cell fates, and later to a region close to the anterior, where it specifies the dorsal-ventral axis (Gonzalez-Reyes et al., 1995; Roth et al., 1995). Paracrine activation of the EGFR by Gurken is, therefore, the first stage of dorsal determination. It is sufficient to establish the dorsoventral axis, since embryos emerging from eggs in which this first step occurs have normal axes. This initiation step also triggers the amplification of signaling: without it, no Rhomboid is expressed (Ruohola-Baker et al., 1993; Hsu et al., 1996) implying, we suggest, that Spitz cannot act. Furthermore, no Vein is expressed in the absence of Gurken/EGFR signaling. The earlier role of the EGFR in posterior determination does not appear

Discussion

Figure 6. Transition from One to Two Peaks of EGFR Signaling

(A and B) In wild-type egg chambers at stage 10, staining for the activated form of MAP kinase reveals a single central domain of activation (arrows). (B) A lateral view of this egg chamber shows that the staining is restricted to the follicle cells adjacent to the anterior of the oocyte (in this view, dorsal is to the left). (C) By stage 11, two peaks (arrows) displaced from the midline can be seen. (D) An egg chamber from an EGFR hypomorph (Egfr<sup>NN1</sup>/Egfr<sup>NN1</sup>). Eggs of this phenotype form a single fused appendage (like that in Figure 1F), and only a single domain of MAP kinase activation is observed.

centered on the dorsal midline (Figures 6A and 6B). By stage 11, two domains, one each side of the dorsal midline, are observed; from their position these cells correspond to the cells that will form the dorsal appendages. In EGFR hypomorphs, which have a fused appendage phenotype (see Figure 1F), the single peak of activated MAP kinase does not split into two (Figure 6D). These results clearly demonstrate that EGFR signaling does indeed evolve from a single peak into twin peaks of activation.

This is supported by examining the expression pattern of known EGFR target genes in the follicle cells. These targets (pointed, rhomboid, argos, and Broad) are expressed in two dorsal anterior domains, one on each side of the midline, by stage 11 (Ruohola-Baker et al., 1993; Morimoto et al., 1996; Musacchio and Perrimon, 1996; Deng and Bownes, 1997; Queenan et al., 1997) (also see Figures 2E, 2F, and 4B). We take this as additional evidence for twin peaks of EGFR activation. Pointed, rhomboid, and argos are also detectable in a single peak at the dorsal midline earlier (see, for example, Figure 4A).

A Distinction between Axis Specification and Eggshell Patterning

EGFR signaling specifies the dorsoventral axis and patterns the eggshell. Our results suggest that these two functions are controlled by temporally separate phases of EGFR activation. When amplification and splitting of EGFR signaling do not occur, eggs have only a single, fused appendage (see, for example, Figures 1E, 1F, 1H, 3H, 5A, and 5B). To our surprise, larvae emerge from these eggs at the frequency predicted by Mendelian principles, and those that emerge have no apparent dorsoventral defects (see Experimental Procedures for explanation of expected frequencies). When follicle cell clones of a spitz null are induced, the hatching rate of eggs with fused appendages was 82% of the predicted number (n = 64). Similarly, 104% (n = 96) and 95% (n = 119) of the predicted number of eggs with a single fused appendage hatch from females of genotype spitz<sup>NN1</sup>/+; ras<sup>112</sup>/+; ras<sup>112</sup> and vn<sup>1067</sup> ras<sup>112</sup>/ras<sup>112</sup>, respectively. The same is true of eggs with fused appendages caused by follicle cell clones of argos null mutations (not shown).

Schnorr and Berg (1996) observed a similar phenomenon in a range of ras1 hypomorphs. Disruption of the amplifying and splitting process therefore does not perturb dorsoventral axis specification, implying that the initial Gurken signal to the EGFR is sufficient to specify the axis (as has been previously proposed [Schüpbach, 1987]). The subsequent cascade of amplification and splitting then patterns the eggshell.
to require Spitz, as we see no anterior–posterior phenotypes in spitz mutant clones, nor in embryos from mothers with the most extreme reduction of signaling (spz112a; ras112a/ras112a). Moreover, Rhomboid is not detectable in the posterior follicle cells. Similarly, Vein appears not to participate in posterior specification. There is an obvious reason that could explain this distinction between anterior–posterior specification and dorsal–ventral specification: the former requires only the determination of a point (the posterior), whereas the latter is more elaborate, starting with determination of the dorsal point but then requiring the patterning of the appendages.

Others have shown that one consequence of Gurken activation of the EGFR is the expression of Rhomboid (Ruohola-Baker et al., 1993; Hsu et al., 1996; Manto and Hsu, 1998). EGFR signaling leads to the loss of the transcriptional repressor CF2 thereby derepressing rhomboid expression. The role of Rhomboid in these, the EGFR-expressing cells, has been a mystery. In other tissues, Rhomboid is thought to regulate the cleavage of Spitz, and it is therefore expected to be present and required in the signal-producing cells, not the signal-receiving cells (for review see Schweitzer and Shilo, 1997; Wasserman and Freeman, 1997). We propose an explanation for Rhomboid’s function in oogenesis: it triggers autocrine Spitz activation of the EGFR, the second stage of dorsal determination. As in other tissues, Rhomboid is essential for the efficient activation of the receptor by Spitz. Since Spitz, Rhomboid, and the EGFR are all present in the follicle cells, however, we cannot address whether Rhomboid acts to process the ligand or enhance its reception.

The autocrine amplification of a paracrine inductive signal is a novel regulatory strategy; why might have it evolved? First, by stage 10/11 of oogenesis, the impermeable vitelline membrane forms and constitutes a physical barrier to signaling between the oocyte and the follicle cells (Mahowald, 1972; Mahowald and Kambsells, 1980). Based on the expression pattern of genes that are responsive to EGFR signaling, it appears that the receptor remains activated when oocyte-derived Gurken can no longer have access to the receptor. The autocrine mechanism described overcomes this difficulty as it occurs outside the vitelline membrane. Second, it is not known if Gurken, like Spitz, requires cleavage. It is possible that uncleaved Gurken initiates the paracrine signal but that diffusible Spitz and Vein allow the signaling to spread more widely. Third, signaling by Gurken alone is insufficient to activate args expression efficiently. Instead, threshold levels of signaling are only achieved after Spitz-mediated amplification, thus activating the Argos/Rhomboid/Spitz/Vein cascade that gives the signaling system its pattern-forming abilities.

Reshaping EGFR Activation with an Autoregulatory Loop

We propose that the third stage of dorsal determination is the reshaping of the profile of EGFR activation. The initial Gurken signal leads to a single peak of activation that establishes the dorsal-most point of the follicle cells, thereby setting the dorsoventral axis of the egg and consequently the embryo. Spitz amplifies, but does not shift, the peak position. The consequence expression of Argos, centered on the midline, radically alters the profile of receptor activation by splitting it. Twin peaks of receptor activation, symmetrically displaced from the midline, are now formed, and in our view these peaks specify the follicle cells that will become dorsal appendages. Interestingly, Morimoto et al. (1996) have previously suggested that the transcription factor Pointed could downregulate the effects of EGFR signaling at the dorsal midline. This result would fit well with our proposal if Pointed acts as an inhibitor by inducing args expression. In fact, extra args expression is seen when Pointed is overexpressed in follicle cells with one of the Gal4 drivers used by Morimoto et al., supporting this idea (J. D. W. and M. F., unpublished data).

It was recently shown that expressing high levels of a ligand-independent activated form of the EGFR causes cells to adopt an operculum-like fate, not dorsal appendages (Queenan et al., 1997), an observation which initially appears inconsistent with our proposal. In this experiment, however, the activated receptor was expressed in the presence of wild-type receptors. When the activated receptor was expressed in gurken mutants, the phenotype was considerably weaker, implying...
that the wild-type receptors contributed significantly to the overall phenotype. In addition, argos was shown to be overexpressed in these eggs, so net EGFR signaling may have been lower than expected. Another complicating factor is the loss of normal temporal regulation. The activated receptor was expressed ectopically by an enhancer that induced precocious signaling, illustrated by first a single peak of EGFR signaling and then its splitting into two. This could dramatically affect the fate of cells receiving the signal.

Our work does not address what specifies the anterior-posterior position of the dorsal appendages, but this seems to be a function of a Dpp signaling gradient with a high point at the anterior-most follicle cells (Twombly et al., 1996; Deng and Bownes, 1997). The position of the appendages is therefore specified by the intersection between EGFR signaling and a presumed A-P gradient of Dpp. The existence of a separate A-P patterning mechanism was confirmed by Queenan et al. (1997) in their experiments with ectopically activated EGFR.

Pattern Formation by Argos

It is easy to see how inductive signaling can trigger the differentiation of a prepatterned structure, but can an initially simple signal be regulated to specify a more complex pattern? In the Drosophila egg chamber it now appears that an autoregulatory network exists that allows Argos to cause the evolution of a point source of EGFR activation into two peaks of signaling, which thereby pattern the egg. Incidentally, there must come a time when follicle cells become refractory to further signaling; otherwise the positive and negative feedback loops would continue to evolve pattern beyond the observed endpoint.

Argos inhibition of the EGFR is also crucial in patterning other tissues. For example, in the eye, Argos mediates a process of "remote inhibition" in which cells at a distance from a source of Spitz are prevented from being induced (Freeman, 1996, 1997). Argos seems to play a related, but slightly different, role in the egg: here, Argos inhibits EGFR signaling locally. This difference is probably explained by variations in how long it takes cells to be irreversibly committed to a particular fate: if cells near the Argos source are already committed (as postulated in the eye) they will not be affected by Argos, although more distant cells will. If, on the other hand, the cells at the Argos source are not yet committed by the time Argos reaches a functional threshold (as we imagine in the follicle cells), they will be the most affected since they are exposed to the highest levels of Argos. The negative feedback loop mediated by Argos is therefore a versatile way of regulating the EGFR in development: distinct spatial regulation is achieved by the same general mechanism, dependent on slightly different local conditions.

Concluding Remarks

In this paper we show that inductive signaling through the EGFR in the oocyte is not a simple paracrine event; instead, the receptor lies at the heart of an integrated network of three activating ligands and an inhibito.
Identical heat shock regimes were administered to control flies, which lacked an FRT sequence on one chromosome but were otherwise genotypically identical to the test flies. In this situation, mitotic recombination does not occur as evidenced by lack of clones in other tissues in the fly.

Sapir et al. (1998) reported that they could not generate follicle cell clones of spitz, args, or rhomboid. They used a selection system that required that eggs with clones survived from the earliest stages of oogenesis. Our system allowed clones to be generated later in development. The early expression of spitz and Egfr suggests that these genes may have much earlier functions in the germarium.

 Germiine clones were generated as previously described (Chou and Perrimon, 1996). Recombination was induced by incubating third-instar larvae or early pupae at 38°C for 1 hr.

Assessment of Viability
To assess viability of embryos contained within eggs with D/V patterning defects, females of the appropriate genotype were crossed to sibling or wild-type males. Eggs with fused appendages were selected, and the number of hatching larvae was determined as a fraction of the total number of fertilized eggs 2-3 days later. When crossed to wild-type males, 100% of fertilized eggs should hatch if eggshell patterning defects do not affect embryonic viability. When crossed to males carrying the same lethal mutation, Mendelian principles would predict 75% hatching.

Acknowledgments

We thank Richard Smith for his excellent technical help, Acaimo Gonzalez-Reyes and Trudi Schüpbach for helpful discussions, Francesca Perl and Siegfried Roth for help with activated MAP kinase staining, Kathy Matthews and Kevin Cooke at the Bloomington Drosophila Stock Centre for many valuable shipments, those people listed in the Experimental Procedures who supplied us with materials, and Tanita Casci, Mariann Bienz, and Sean Munro for advice on the manuscript. This work was partly supported by the UK-Israel Science and Technology Research Fund.

Received May 18, 1998; revised August 27, 1998.

References


