The Transmembrane Molecule Kekkon 1 Acts in a Feedback Loop to Negatively Regulate the Activity of the Drosophila EGF Receptor during Oogenesis

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Summary

We have identified the Drosophila transmembrane molecule kekkon 1 (kek1) as an inhibitor of the epidermal growth factor receptor (EGFR) and demonstrate that it acts in a negative feedback loop to modulate the activity of the EGFR tyrosine kinase. During oogenesis, kek1 is expressed in response to the Gurken/EGFR signaling pathway, and loss of kek1 activity is associated with an increase in EGFR signaling. Consistent with our loss-of-function studies, we demonstrate that ectopic overexpression of kek1 mimics a loss of EGFR activity. We show that the extracellular and transmembrane domains of Kek1 can inhibit and physically associate with the EGFR, suggesting potential models for this inhibitory mechanism.

Introduction

The biological processes regulated by receptor tyrosine kinase (RTK) signaling pathways are diverse and include the regulation of cell growth, differentiation, migration, viability, and maintenance of homeostasis (see review by Ullrich and Schlessinger, 1990). In the past few years, a great deal has been learned about the mechanisms by which RTKs transduce signals and specify particular cellular fates. In particular, most RTKs have been shown to regulate gene expression through the Ras/Raf/MEK/MAPK cassette (see Li and Perrimon, 1997). Although the pathways that transduce signals from RTKs to the nucleus have been well characterized, less is known about the mechanisms involved in imparting specificity. More recently, it has been shown that modulation of RTK activities is a critical aspect of their biological roles, since quantitative variations in their level of activation can lead to very different cellular outcomes. For example, studies in mammalian PC12 cells have illustrated that prolonged MAPK activation and nuclear translocation triggers differentiation, whereas transient activation leads to proliferation (see Li and Perrimon, 1997). Similarly, in the Drosophila embryos, patterning of the terminal region is determined precisely by the strength of the signal generated by the Torso RTK (Greenwood and Struhl, 1997). Thus, precise regulation of the quantitative output of a signal generated by an RTK is critical to the control of growth and differentiation.

While many positive regulators/effectors of RTK signaling activity have been characterized, only a few inhibitory molecules have been identified (Freeman et al., 1992; Kharitonenko et al., 1997). In addition to these inhibitory molecules, a number of inhibitory mechanisms have been identified. These include internalization of ligand–receptor complexes, receptor desensitization as a result of hyperphosphorylation of the receptor, inhibitory regulation by cytoplasmic and transmembrane protein phosphatases, and targeting to the proteolytic pathway of specific signal transducers (Ullrich and Schlessinger, 1990; Sturtevant et al., 1994; van der Geer et al., 1994; Kokel et al., 1998). By integrating particular stimulatory and inhibitory mechanisms in cells, RTK activities can be quantitatively regulated to direct distinct cellular outcomes.

The Drosophila EGFR is required for a multitude of developmental processes throughout the life cycle (reviews by Ray and Schupbach, 1996; Perrimon and Perkins, 1997; Schweitzer and Shilo, 1990). For example, activation of the EGFR pathway in follicle cells during oogenesis establishes both anteroposterior and dorsoventral fates. During embryogenesis, the EGFR is required for the establishment of ventral cell fates, determination of segmental identity, maintenance of amnioserosa and ventral neuroectodermal cells, germband retraction, cell fate specification in the central nervous system, and production of cuticle. During larval development, the EGFR plays a role in cell proliferation, vein formation, and eye development. These multiple tissue-specific activities are regulated in part by different ligands that contain an EGF repeat similar to that of transforming growth factor α (TGF-α), a known ligand of the vertebrate EGFR. To date, three such ligands have been isolated in Drosophila: Spitz (Spi; Rutledge et al., 1992; Schweitzer et al., 1995a), Gurken (Grk; Neuman-Silberberg and Schupbach, 1993), and Vein (Vn; Schnepp et al., 1996). In contrast to these stimulatory molecules, Argos (Aos), a secreted protein with an atypical EGF motif, has been shown to act as an antagonist of EGFR activity (Schweitzer et al., 1995b). In vitro studies have revealed that Aos can interfere with EGFR activation in a manner that is both saturable and competitive, and it has been proposed that Aos acts as a direct inhibitor of Spi by binding directly to the EGFR (Schweitzer et al., 1995b). Remarkably, the expression of aos is dependent upon EGFR activation (Golembo et al., 1996b), thus demonstrating

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that the EGFR regulates the expression of its own negative regulator.

During late oogenesis, EGFR activity is regulated by all of these mechanisms, and its precise activity is critical in specifying the proper pattern of the chorion and embryo (review by Ray and Schupbach, 1996). Thus, function of the EGFR during oogenesis provides an excellent paradigm to characterize the quantitative regulation of the EGFR and determine if additional components of this pathway exist. In response to Grk signaling from the oocyte, activation of the EGFR in follicle cells follows a dynamic pattern. In early egg chambers, Grk first activates the EGFR pathway in posterior follicle cells. Subsequently, around stage 7, grk transcripts become localized to the anterodorsal corner of the oocyte and signal to adjacent follicle cells to define their dorsal fates. Activated EGFR molecules regulate transcription through the Ras/Raf/MEK/MAPK cassette in follicle cells (Brand and Perrimon, 1994; Hsu and Perrimon, 1994; Schnorr and Berg, 1996). In a screen to identify downstream genes regulated by the EGFR in follicle cells, we have identified the gene kekkon 1 (kek1), which encodes a single pass transmembrane protein with features of a cell adhesion molecule with leucine-rich repeats (LRR) and immunoglobulin (Ig) motifs (Musacchio and Perrimon, 1996), as a target gene. We demonstrate that endogenous kek1 acts in an inhibitory manner: loss of kek1 activity results in increased EGFR signaling. We also demonstrate that overexpression of kek1 blocks the activity of the EGFR. This inhibition involves a physical association between the extracellular and transmembrane domains of Kek1 with the EGFR. This establishes Kek1 as a bona fide inhibitor of the EGFR that acts in a negative feedback loop to modulate the activity of this RTK signaling pathway.

Results

kek1 Is Regulated by the Grk/EGFR Pathway in Follicle Cells

To characterize target genes of the Grk/EGFR signaling pathway, we screened a collection of enhancer trap lines for expression patterns in follicle cells. We found one line, 15A6, which was expressed in a pattern consistent with it being a regulatory target of the EGFR pathway, since it undergoes a transition during stages 8-10 when it relocates from a posterior to a dorsal-anterior gradient (Figures 1A–1C). 15A6 corresponds to an insertion in the kek1 gene (Musacchio and Perrimon, 1996), and we refer to it as kek-lacZ in the text. We found that kek-lacZ expression reflects accurately the expression of kek1 both in wild-type (WT) and in mutant backgrounds (data not shown).

To establish a regulatory link between the transcriptional regulation of kek1 in the follicle cell epithelium and the Grk/EGFR signaling pathway, we examined kek-lacZ expression in mutants for grk, egfr, and the kinase Draf that is both necessary and sufficient for EGFR signaling in follicle cells (Brand and Perrimon, 1994). In each case, the dorsal-anterior gradient of kek-lacZ expression was abolished or severely diminished (Figures 1D and 1E; see also Brand and Perrimon, 1994; Queenan et al., 1997; Sapir et al., 1998), revealing that kek-lacZ expression is transcriptionally regulated by the Grk/EGFR/Draf pathway. Expression of an activated form of Draf resulted in the ubiquitous expression of kek-lacZ within the follicular epithelium (Figure 1F).

To further analyze the role of the Grk/EGFR pathway in the transcriptional control of kek-lacZ, we mislocalized the subcellular localization of grk mRNAs by disrupting the oocyte cytoskeletal network (Figure 1G).
and (A4), (B4), and (C4) are lateral and ventral views, respectively. In all panels, anterior is to the left. The chorions shown in (A1), (B1), and (C1) are dorsal views. The embryos in (A2), (B2), (C2), (A3), (B3), (C3), and (A4), (B4), and (C4) are lateral and ventral views, respectively.

Treatment of the oocyte with colchicine, an inhibitor of microtubule polymerization, leads to mislocalization of the oocyte nucleus and its associated grk mRNAs, which correlated with ectopic kek-lacZ expression (Figure 1H) as well as kek1 mRNA expression (not shown). Altogether, these results demonstrate that Grk is not only necessary but sufficient to regulate the spatial expression of kek1.

Overexpression of kek1 during Oogenesis Antagonizes EGFR Activity

Previous analysis has revealed that in the complete absence of kek1 gene activity, flies are viable, fertile, and do not exhibit any overt morphological defects (Musacchio and Perrimon, 1996). Kek1 has the features of cell adhesion molecules (CAMs), and loss-of-function mutations of many CAMs have subtle mutant phenotypes. However, when ectopically overexpressed, some CAM molecules can generate striking mutant phenotypes that are revealing of their functions (Speicher et al., 1998). Thus, to gain insights into the possible function of kek1 during oogenesis, we tested the effect of overexpressing kek1 in the follicle cells using the GAL4/UAS system (Brand and Perrimon, 1993).

When kek1 is expressed under the control of the GAL4 line T155, which drives expression all over the follicle cell epithelium beginning at stage 9 (Harrison et al., 1995; Queenan et al., 1997), 100% of the resulting eggs are longer than the WT eggs and have a reduction or a complete loss of dorsal appendages (cf. Figure 2B1 with the WT in Figure 2A1). This loss of dorsal appendage material is due to a ventralization of the eggshell, a phenotype associated with egrf, or grk loss-of-function mutations (Schupbach, 1987; Price et al., 1989). A similar ventralized eggshell phenotype was obtained when a dominant-negative form of the EGFR, EGFRDN, was expressed under the control of T155 (Figure 2C1).

In addition to its role in establishing the dorsal characteristics of the eggshell, the Grk/EGFR pathway controls embryonic dorsoventral patterning by restricting a ventralizing signal (Neuman-Silberberg and Schupbach, 1994; Morisato and Anderson, 1995). As a result, loss of EGFR function leads to ventralized embryos. Embryonic cuticles derived from T155; UAS-kek1 females showed a ventralized cuticle phenotype (Figure 2B2). To analyze the extent of this ventralization, we stained embryos for Twist (Twi) RNA and protein that label a ventral domain in ten cells wide (Thissie et al., 1988). Overexpression of kek1 in follicle cells results in an expansion of the Twi expression domain (Figures 2B3 and 2B4). Similarly, embryos derived from T155; UAS-egrfDN females are also strongly ventralized (Figures 2C2-2C4).

Altogether, our results indicate that overexpression of kek1 in follicle cells ventralizes both the eggshell and the embryo by most likely interfering with the activity of the Grk/EGFR signaling pathway.

Kek1 Inhibits the Activity of the Ras/Raf/MEK/MAPK Cassette

To determine whether Kek1 blocks the signaling activity of the EGFR, we tested whether ectopic expression of kek1 blocks the transcriptional activation of target genes regulated by the EGFR/Ras/Raf/MEK/MAPK pathway in follicle cells. Since kek1 is a target of this pathway, we used kek-lacZ as a reporter for this experiment. Overexpression of kek1 using the Gal4 driver T155 was associated with a strong but not complete reduction of kek-lacZ expression (cf. Figure 3B to WT in Figure 3A). Using a stronger Gal4 line, CY2 (Queenan et al., 1997), a complete disappearance of kek-lacZ expression was observed (Figure 3C). These data are consistent with the model that Kek1 downregulates the activity of the Ras/Raf/MEK/MAPK pathway. Consistent with this, epistasis experiments indicate that the inhibitory effect of Kek1...
The Extracellular Domain of Kek1 Is Critical for the Inhibitory Effect
To test whether the extracellular domain of Kek1, which contains five LRR and one Ig motif (Musacchio and Perrimon, 1996), is required for the inhibition of the EGFR activity by Kek1, we generated transgenic lines that contain either UAS-kek1extra or UAS-kek1intra.

No phenotype were observed by overexpressing kek1intra (data not shown) in the follicle cells using the T155 or CY2 GAL4 lines. On the other hand, overexpression of kek1extra using the same drivers led to eggs with reduced dorsal appendage materials (Figure 5A) and ventralized embryos (Figures 5B and 5C). These observations indicate that the extracellular domain of Kek1 is sufficient to inhibit the activity of the EGFR.

Although the phenotypes obtained following kek1extra overexpression are very similar to those obtained with full-length kek1, we note that these phenotypes are weaker. One possibility is that Kek1extra is expressed at a lower level than the WT protein. Weaker effects were observed with 12 independent transgenic lines, as well as when multiple copies of the transgenes were added. This suggests that Kek1extra is less stable than the full-length Kek1 molecule or that the cytoplasmic domain of Kek1 participates to some extent in the inhibition.

Finally, we tested whether the inhibition of the EGFR activity by kek1extra requires the presence of the endogenous Kek1 protein. When kek1extra was overexpressed in kek1mutant females, the eggs were ventralized, which excludes a model whereby kek1extra blocks the EGFR by forming a heterodimer with the endogenous Kek1 protein (data not shown).
Kek1 Is a Negative Regulator of the Grk/EGFR Signaling Pathway

Because of the severe inhibition of EGFR activity by Kek1 revealed by the overexpression experiment, we decided to reexamine the effect of loss of kek1 function during oogenesis. We reasoned that a subtle egg morphology phenotype may have been missed by simply using fertility as an assay (Musacchio and Perrimon, 1996). Indeed, we found that the spacing between the dorsal appendages of eggs derived from kek1 mutant females was increased when compared to WT. Further, these eggs were also mildly shorter and rounder (Figure 6A1), a phenotype consistent with a hyperactivation of the Grk/EGFR pathway (Neuman-Silberberg and Schupbach, 1994). These features do not interfere with hatching rates and patterning that is consistent with the normal Twi expression found in kek1 mutant embryos (n = 142). Interestingly, when kek1 mutant flies were raised at 29°C, 5% (n = 257) of the embryos derived from kek1 mutant females showed a mild reduction in Twi expression (Figures 6A2 and 6A3). We conclude that loss of kek1 activity during oogenesis leads to mildly dorsIALIZED eggs.

To substantiate the functional relationship between Kek1 and the Grk/EGFR signaling pathway during oogenesis, we examined the effects of a loss of Kek1 activity in two different genetic backgrounds. First, we tested if an increase in the level of Grk molecules can generate a stronger phenotype in the absence of kek1. Previously, Neuman-Silberberg and Schupbach (1994) showed that females carrying four copies of a transgene expressing grk ([P[grk]], in addition to the two endogenous copies, lay a significant fraction of partially or severely dorsIALIZED eggs. In contrast, females that carry only one copy of [P[grk]] lay WT eggs (Neuman-Silberberg and Schupbach, 1994; this study, data not shown). We found that eggs laid by females carrying one copy of [P[grk]] in the absence of kek1 are significantly dorsIALIZED (Figure 6B1): the spacing between the dorsal appendages is increased and the eggs are rounder and smaller. Further, Twi expression that is normally expressed in embryos laid by females carrying one copy of [P[grk]] (n = 168; data not shown) is repressed ventrally in 15% (n = 181) of the embryos derived from kek1 mutant females that carry a single copy of [P[grk]]. When raised at 29°C, 22% (n = 348) of the embryos derived from eggs laid by females carrying one copy of [P[grk]] in the absence of kek1 showed a dorsIALIZED phenotype readily detectable by aberrant Twi expression. We note that there is a variability in the reduction of the Twi domain, ranging from embryos in which Twi-expressing cells are missing in the middle regions of the embryo to the most severe cases in which only the poles express the Twi protein (Figures 6B2 and 6B3).

Second, we reasoned that if loss of Kek1 activity leads to a hyperactivation of EGFR activity, then loss of Kek1 activity should rescue a decrease in EGFR activity. We tested this hypothesis by generating flies that simultaneously lacked Kek1 activity and had reduced activity of the EGFR. Consistent with our hypothesis, loss of Kek1 activity resulted in partial suppression of the EGFR phenotype (Figures 6C1-6C3). Eggs laid by females mutant for EGFR alone have a ventralized phenotype (Schupbach, 1987). They exhibit either no or only a single dorsal appendage, and the eggs are significantly longer than WT (100%, n = 852; Figures 6C1 and 6C2). In contrast, the majority of eggs (95.5%, n = 1103) laid by the kek1+egfr double mutant females have two dorsal appendages and their length is shorter, consistent with a reversion to a more WT phenotype (Figure 6C3).

Kek1 Physically Associates with the EGFR

Having demonstrated that the in vivo role of kek1 is consistent with the results obtained by overexpression, we wanted to gain insight into the mechanism of inhibition of the EGFR by Kek1. To do so, we tested whether EGFR and Kek1 physically associate. Following coexpression of a Myc-tagged version of Kek1 and the EGFR in Sf9 cells, Kek1 was immunoprecipitated from the cell lysates using an anti-Myc antibody. Coprecipitation of the EGFR was observed by probing the resulting blot with the anti-EGFR antibody (Figure 7A), suggesting that Kek1 associates physically with the EGFR and that this interaction is responsible for the inhibitory effect. When both Kek1 and Torso were coexpressed in Sf9 cells, no Torso was coprecipitated by Kek1 (Figure 7A), suggesting a selectivity of Kek1 for binding to the EGFR.

Because the extracellular and transmembrane portions of Kek1 are essential for the inhibitory effect, we examined whether these portions of Kek1 are able to bind to the EGFR. As shown in Figure 7B, the extracellular domain, but not the intracellular domain, of Kek1 can bind to the EGFR. Thus, we propose that the inhibitory effect of the EGFR by Kek1 is mediated through direct association of the extracellular and transmembrane domains of Kek1 with the EGFR.
Figure 6. Phenotypes Associated with Loss of kek1 Function during Oogenesis

(A1-A3) At 25°C, eggs derived from kek1 mutant females showed a weak dorsalization phenotype that does not interfere with fertility. This dorsalization phenotype is evident by the more lateral position of the dorsal appendages (cf. Figure 6A1 with a WT eggshell shown in Figure 2A1). By increasing the temperature, we observed a weak enhancement of both the eggshell and the embryonic phenotypes readily detectable by aberrant Twi expression (Figures 6A2 and 6A3).

(B1-B3) The dorsalization phenotype associated with loss of kek1 function is enhanced by an increase in the level of Grk ligand. The eggs derived from kek1 mutant females that contain an extra copy of grk, provided by a P[grk] transgene, showed an enhancement of the dorsalized eggshell phenotype (cf. Figure 6B1 with Figure 6A1), and the embryos derived from these eggs showed a stronger dorsalized phenotype (cf. Figures 6B2 and 6B3 with Figures 6A2 and 6A3). Orientations are the same as in Figure 2.

(C1-C3) Suppression of the egg phenotype associated with egrf mutations by kek1. Chorions derived from egrf or kek1 egrf females are shown. (C1) egrfQY1/egrf2E07. (C2) egrfQY1/kek1RM2 egrf2E07. (C3) kek1RA5 egrfQY1/kek1RM2 egrf2E07. In addition to the recovery of the two dorsal appendages, the length of the chorion has also become similar in length to WT.

Discussion

We have identified the transmembrane protein Kek1 as a molecule that acts in a negative feedback loop to modulate the activity of the Drosophila EGFR tyrosine kinase during oogenesis. We provide loss-of-function, ectopic overexpression, and biochemical evidence to support this. First, eggs laid by females that lack kek1 gene activity are weakly dorsalized, and loss of kek1 gene activity can suppress egrf mutations and potentiate Grk signaling. Second, ectopic overexpression of Kek1 in follicle cells leads to severe ventralization identical to effects seen in EGFR mutants, providing further support of Kek1 as a negative regulator of the EGFR pathway. Finally, we provide biochemical evidence that the inhibition of EGFR activity by Kek1 involves the direct association between the extracellular and transmembrane domains of Kek1 and the EGFR.

Interestingly, kek1 is expressed in the eye and wing imaginal discs in a pattern that is highly suggestive of induction by the EGFR (Musacchio and Perrimon, 1996). Although no obvious phenotypes have been described in these tissues in kek1 mutants, we found that overexpression of kek1 in these tissues also generate phenotypes reminiscent to loss of EGFR activity (not shown). This suggests that the negative regulation of the EGFR by Kek1 is not only restricted to oogenesis.

The Function of Kek1 in Patterning of the Follicle Cell Epithelium

During oogenesis, Grk derived from the oocyte activates in a paracrine fashion the EGFR in dorsal follicle cells. Recent work from Wasserman and Freeman (1998) has shown that this paracrine signaling leads to the activation of a second phase of signaling, whereby the EGFR activity is amplified among follicle cells themselves. During this second phase, the EGFR activates a number of target genes that include both positive (rho and vn) and negative (aos) regulators of the pathway. Activation of Rho in follicle cells presumably leads to the activation of the Spi EGFR ligand, while activation of Aos within the peak of EGFR activity at the dorsal anterior leads to repression of EGFR, effectively splitting the initial peak of EGFR activity into two. This splitting of EGFR activity eventually defines the domains where the dorsal appendages will form.

Our identification of Kek1, together with the studies on Aos, indicates that there are at least two different negative regulators of EGFR activity in follicle cells. However, the regulation and function of Kek1 is distinct from those of Aos. aos is expressed only in response to high levels of EGFR activity, while kek1 is expressed in a graded fashion. Further, loss-of-function phenotypes of kek1 and aos in follicle cells are different. In the absence of kek1 activity, the spacing between the dorsal appendages is increased, while in the absence
Further, we have shown that the extracellular and transmembrane domains of Kek1 are sufficient for this inhibition. The extracellular domain of Kek1 contains one Ig-like domain and five LRRs, both of which can mediate protein-protein interactions (Musacchio and Perrimon, 1996). A number of mechanisms can underlie the mechanism by which this extracellular domain acts as an inhibitor. For example, the Kek1 extracellular domain could mask the accessibility of the extracellular domain of the EGFR to all ligands. Conversely, it could form a heterodimer with EGFR monomers and block their dimerization. Dimerization is a prerequisite to the activation of downstream signaling events by the RTK. Alternatively, Kek1 could be involved in bringing a transmembrane tyrosine phosphatase to the vicinity of the EGFR and thus lead to its deactivation. Consistent with these models, we have observed that Kek1 can inhibit mammalian EGFR molecules from becoming tyrosine phosphorylated in response to growth factor treatment in infected insect cells (L. T. A. et al., unpublished observations). Finally, we envision that Kek1 could target the EGFR to a degradation pathway through endocytosis or bind to additional proteins involved in their subcellular localization. Studies on the C. elegans LET-23 EGFR have well illustrated the critical role of subcellular localization and PDZ proteins in signaling by this RTK (Kim, 1997; Kaech et al., 1998). Interestingly, we note that both Kek1 and the EGFR contain a TXV motif at the C terminus. This S/TXV motif has been implicated in protein-protein interactions and suggests that Kek1 of aos, the appendages are fused dorsally. Aos has been proposed to split the initial peak of EGFR activity into two (Wasserman and Freeman, 1998). We propose that the function of Kek1 is to restrict the lateral spreading of EGFR activation by Spi. Thus, in the absence of kek1 activity, Rho/Spi activation could spread more laterally, explaining the enhancement of the spacing between the two dorsal appendages.

**Mechanism of Inhibition of the EGFR by Kek1**

Our analysis demonstrates that Kek1 acts as a potent negative regulator of EGFR activity when overexpressed.
as inhibitors of secreted Wnt ligands (Leyns et al., 1997; Wang et al., 1997). Finally, not all negative regulators that act in feedback loops act extracellularly. For example, Smad7, which is expressed in response to TGF-β signaling, blocks the activity of this pathway intracellularly by binding to the TGF-β receptor and by inhibiting Smad2 and Smad3 phosphorylation that is required for transmission of the signal (Nakao et al., 1997). Another example is the human CL100 phosphatase that after being expressed following exposure to either oxidative stress or heat shock downregulates specifically the MAP kinase (Keyse and Emslie, 1992). The Drosophila homolog of CL100, known as puckered, has been shown to mediate a feedback loop regulating JNK activity during dorsal closure in Drosophila (Martin-Blanco et al., 1998).

Kek1 Defines a Novel Gene Family

In Drosophila, two other putative Kek molecules that share extensive homologies with kek1 have been identified: kek2 (Musacchio and Perrimon, 1996) and kek3 (our unpublished data). The function of these additional Kek-like proteins is not known. However, it is interesting to note that despite extensive saturation of the region containing kek3, no mutant alleles have been recovered (Ashburner et al., 1990; Spradling et al., 1995; our unpublished data). Whether or not this and the subtle effect of loss of Kek1 activity is due to redundancy within the Kekkon family remains to be determined. Thus, it will be important to characterize the expression patterns as well as the loss-of-function and overexpression phenotypes of kek2 and kek3 in order to evaluate any potential abilities to modulate the EGFR activity. Further, chimeric proteins between these molecules may help to further define the Kek1 domain(s) required for the inhibitory mechanism.

Finally, we note that there are putative transmembrane proteins in vertebrates (Suzuki et al., 1996) and invertebrates that show similar arrangements of LRRs and Ig motifs. This raises the possibility that Kek1 is a member of a family of structurally related EGFR inhibitors. As alteration in the activity of the various members of the human EGFR/ErbB family has strong links to oncogenesis, it will be important to determine if vertebrate LRR/Ig molecules share functional, in addition to sequence, similarities to Kek1. We anticipate that the continued characterization of the Kek and related molecules will provide novel approaches to the design of inhibitors of the EGFR/ErbB family for therapeutic use in oncogenesis.

Experimental Procedures

Fly Stocks

Various collections of enhancer trap lines were screened for expression patterns within the follicular epithelium (Perrimon et al., 1991; Smith et al., 1993; D. Eberl, unpublished). From this screen, the second chromosomal enhancer trap line 15A6 was identified. For a description of 15A6, which we refer to as kek-lacZ, see Musacchio and Perrimon (1996). The two overlapping deficiencies, RA5 and RM2, completely delete the kek1 gene (Musacchio and Perrimon, 1996), and we refer to RA5/RM2 females as kek1 mutant females in this paper.

The alleles of the egfr, egfrV1, egfrV2, egfrwat, egfrdaz are described in Clifford and Schupbach (1989). For a description of grkexon, see Schupbach (1987) and Neuman-Silberberg and Schupbach (1993). The hypomorphic Draf mutation Drafad has been described in Melnick et al. (1993). hs-Drafac, which expresses an N-terminal truncation of Draf is described in Brand and Perrimon (1994). The UAS-EGFRDN is described in Freeman (1996). The enhancer trap line es379, the Pgrkr, and the UAS-Atop (Queenan et al., 1997) files were a gift from T. Schupbach.

Ectopic expression in the follicular epithelium was performed with the pGawB GAL4 lines T155 (Harrison et al., 1995) and CY2 (Queenan et al., 1997).

Inhibitor Studies

The colchicine inhibitor was used at 25 or 50 μg/ml to destabilize microtubules as described in Theurkauf et al. (1993). As a control, flies were treated with 50 μg/ml of β-lumicolchicine, which is an inactive isomer of colchicine. After initiating treatment, flies were dissected and fixed at 4, 8, 10, and 24 hr intervals over 48 hr and probed for expression of grk mRNAs. Stage 10 chambers treated after the dorsal-anterior migration of the oocyte nucleus showed a higher frequency of disrupted and irregular kek-lacZ expression (49/156) than chambers treated prior to the migration (17/108). Earlier treated chambers had a higher frequency of posteriorly shifted gradients (88/108 early versus 31/156 late). Finally, we detected no effects with cytochalasin D, an inhibitor of microfilaments.

In Situ Hybridization and β-Galactosidase Histochemistry

In situ hybridization was performed with digoxigenin-labeled grk, kek1, and twist RNA essentially as described in Tautz and Pfeifle (1989) using 55°C and 65°C as the hybridization temperature.

For detection of β-galactosidase activity, ovaries were dissected in PBT (PBS + 0.1% Tween 20) and fixed in 4% methanol-free formaldehyde for 15 min or in 2.5% glutaraldehyde for 2.5 min. Ovaries were then stained with 1 mg/ml X-Gal in X-Gal staining buffer at 25°C for 3.5-4.5 hr.

The polyclonal rabbit anti-Twist antibody (Roth et al., 1989) was used at a dilution of 1:5000.

For visualization, embryos and egg chambers were directly mounted in 80% glycerol and photographed under Nomarski optics with a Zeiss Axioskop microscope. Embryonic cuticles and chorions were prepared according to van der Meer (1977) and visualized using dark-field optics.

Constructs

UAS-kek1 was made in the following manner. A HindIII/EcoRI fragment from the kek1 cDNA containing the complete coding region (Musacchio and Perrimon, 1996) was subcloned into pB5K'. From this subclone, an XbaI/XhoI fragment containing the complete kek1 coding region was cloned into the P element vector pUAST (Brand and Perrimon, 1993). UAS-kek1-exon was made by inserting by PCR a stop codon immediately after the transmembrane domain of kek1 and UAS keklacZ by fusing a v-Src myristylation site to the entire intracellular domain of kek1. P element-mediated transduction was performed following injection into the delta 2-3 transposase strain (Robertson et al., 1988).

Insect Cell Experiments

cDNAs encoding the Drosophila EGFR and myc-tagged versions at the C terminus of full-length kek1, keklacZ, and kek1exon were subcloned into the baculovirus transfer vectors pVL1392 or pVL1393 (Pharmingen).

Recombinant baculoviruses were produced in SF9 insect cells using the Bac-N-Blue transfection kit (Invitrogen) and plaque purified prior to use. For coexpression experiments, 10⁵ SF9 cells were infected at a multiplicity of infection of 30. For the EGFR/Torso comparison experiment, cells were infected with a 1:1:28 ratio of Drosophila EGFR:Kek1:WT baculovirus. For the domain deletion experiment, cells were infected with a 10:20 ratio of EGFR:Kek1 virus. For both experiments, cells were infected for 48 hr and then lysed in 1 ml 20 mM HEPES/Na, 150 mM NaCl, 1mM EDTA, 1% NP-40, 1 mM PMFS, and 1 mg/ml each of pepstatin A, aprotinin, and leupeptin. Cleared lysates were immunoprecipitated with 1 μg anti-Myc (Ab2, NeoMarkers). Precipitates were washed three times with lysis buffer, resolved by 6% SDS-PAGE, and blotted with 1/5000
anti-EGFR or 1/5000 anti-Torso. Filters were then stripped and re-probed with 1/200 anti-Myc.

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