Knockouts of Kekkon1 Define Sequence Elements Essential for Drosophila Epidermal Growth Factor Receptor Inhibition

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ABSTRACT

Throughout development, cells utilize feedback inhibition of receptor tyrosine kinase (RTK) signaling as an important means to direct cellular fates. In Drosophila, epidermal growth factor receptor (EGFR) activity is tightly regulated by a complex array of autoregulatory loops, involving an assortment of inhibitory proteins. One inhibitor, the transmembrane protein Kekkon1 (Kek1) functions during oogenesis in a negative feedback loop to directly attenuate EGFR activity. Kek1 contains both leucine-rich repeats (LRRs) and an immunoglobulin (Ig) domain, two of the most prevalent motifs found within metazoan genomes. Here we demonstrate that Kek1 inhibits EGFR activity during eye development and use this role to identify *kek1* loss-of-function mutations that implicate the LRRs in directing receptor inhibition. Using a *GMR-GAL4, UAS kek1-GFP* misexpression phenotype we isolated missense mutations in the *kek1* transgene affecting its ability to inhibit EGFR signaling. Genetic, molecular, and biochemical characterization of these alleles indicated that they represent two functionally distinct classes. Class I alleles directly diminish Kek1's affinity for EGFR, while class II alleles disrupt Kek1's subcellular localization, thereby indirectly affecting its ability to associate with and inhibit the receptor. All class I alleles map to the first and second LRRs of Kek1, suggesting a primary role for these two repeats in specifying association with and inhibition of EGFR. Last, our analysis implicates glycine 160 of the second LRR in regulating EGFR binding.

SIGNALING by the epidermal growth factor receptor (EGFR) plays a critical role throughout development, where it mediates a wide array of cellular decisions (Schweitzer and Shilo 1997; Dominguez *et al.* 1998; Nilson and Schupbach 1999; Van Buskirk and Schupbach 1999). In each developmental pathway, EGFR signaling is subject to both positive and negative regulatory mechanisms and feedback loops to ensure the appropriate cellular response. For example, negative regulators of EGFR signaling, such as Kekkon1 (Kek1), Argos (Aos), D-Cbl, and Echinoid (Ed), differ in their mechanism of function, tissue specificity, or temporal expression and thus contribute to EGFR signaling in different ways to promote distinct phenotypic outcomes (Schweitzer *et al.* 1995; Hime *et al.* 1997; Ghiglione *et al.* 1999; Pai *et al.* 2000; Bai *et al.* 2001). The establishment of dorsal-ventral polarity during oogenesis in Drosophila provides one well-documented example of this, as mutations in each of the inhibitors, Kek1, Aos, and D-Cbl, all display different phenotypes (Stevens 1998; Pai *et al.* 2000). *kek1* knockouts exhibit increased spacing between the dorsal appendages, *aos* mutations result in a single wide appendage, and *D-Cbl* mutations result in complete dorsalization (Stevens 1998; Wasserman and Freeman 1998; Pai *et al.* 2000).

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Consistent with their unique roles in EGFR signaling, each of these inhibitors encodes a structurally distinct molecule. Whereas Aos encodes an inhibitory ligand and D-Cbl is a cytoplasmic inhibitor, Kek1 is a single-pass transmembrane inhibitor (Schweitzer *et al.* 1995; Hime *et al.* 1997; Ghiglione *et al.* 1999; Pai *et al.* 2000; Bai *et al.* 2001). On the extracellular side, Kek1 is composed of an N-terminal insert, seven leucine-rich repeats (LRRs) flanked by cysteine-rich regions, and a single immunoglobulin-like domain (Ig; Musacchio and Perrimon 1996). Current data indicate that the extracellular and transmembrane domains of Kek1 suffice to inhibit EGFR signaling, where the LRRs are necessary for Kek1 function (Ghiglione *et al.* 1999, 2003). In contrast, while the cytoplasmic domain was previously reported to be dispensable for inhibition, it has been recently implicated in Kek1 trafficking (Ghiglione *et al.* 2003). LRRs and Ig domains, which are modules for intermolecular interactions, represent the second most prevalent repeat and domain, respectively, within the Drosophila proteome (Pruess *et al.* 2003). Moreover, on the basis of the presence of these modules, Kek1 is a member of a multigene family that contains five additional members in *Drosophila melanogaster* (Derheimer *et al.* 2004, accompanying article). Throughout the extracellular region these molecules share extensive sequence similarity; however, functional analysis suggests that inhibition of EGFR signaling is unique to Kek1 (Alvarado *et al.* 2004). Given this, one important goal

in defining Kek1 function is to identify sequence elements in Kek1 that direct binding and inhibition of the EGFR.

While deletion studies provide insight into the relevance of each module, the abundance of the LRR and Ig modules in the proteome requires that more detailed analyses of Kek1 would have to be undertaken to identify residues imparting functional specificity. Therefore, to rapidly define residues crucial for Kek1's inhibitory function and to gain insight into the specificity of this interaction, we chose to identify an allelic series of mutations in *kek1*. Initially, we demonstrate by mutational and misexpression analyses that Kek1 inhibits EGFR in the developing eye. We then utilize this role to identify loss-of-function (LOF) missense mutations in *kek1* by screening for suppressors of a *kek1-gfp* misexpression phenotype in the compound eye. From this screen we identified 10 alleles of *kek1* that partially or completely abolished its activity. These lesions map throughout the extracellular domain of Kek1 and can be grouped into two classes on the basis of their effects on subcellular distribution. Class I alleles display normal apical Kek1-GFP localization, while class II alleles localize aberrantly. Co-immunoprecipitation assays revealed that only class I alleles exhibit decreased binding affinity for EGFR, indicating that distinct mechanisms underlie the LOF effects of the two allelic classes. Strikingly, mutations in glycine 160 in the second LRR were independently isolated three times, suggesting that this residue has a crucial role in directing Kek1's interaction with and inhibition of the EGFR.

MATERIALS AND METHODS

Screen for alleles of *kek1*: *P{GAL4-ninaE.GMR}, P{UAS kek1-gfp}/CyO* flies display a severe rough eye phenotype and were generated by standard recombination methods from stocks containing individual P insertions. Males were mutagenized with 25 mM EMS according to previously described methods (Ashburner 1989). Mutagenized males were crossed to *w; iso2; iso3* females and maintained at 25° (Figure 2). A total of 105,000 chromosomes from straight-winged F_1 males and females were screened for suppression of the parental phenotype. From this total, 134 straight-winged suppressors ranging from weak to complete were crossed to *y w; Sco/CyO* flies and balanced over *CyO*. A number of suppressors were not retained in the F_2 due to recombination between both P insertions in the F_1 females, mosaicism of the induced mutation in the F_1, or sterility. F_2 progeny were crossed to *P{UAS-DNegfr}* (Freeman 1996) to test the functional integrity of *P{GAL4-ninaE.GMR}* (Freeman 1996) and to the follicle cell driver *P{GawB}CY2* (Queenan *et al.* 1997) to test for activity of the *P{UAS-kek1-gfp}* responder. Green fluorescent protein (GFP) fluorescence was used as a marker to verify the presence of *P{UAS-kek1-gfp}* and also to identify missense mutations. We recovered 10 suppressors, consistent with mutations in *P{UAS-kek1-gfp}*. To follow the suppression phenotype, both P inserts were maintained as recombinants on the second chromosome and balanced over *CyO*.

To demonstrate that suppression originated from mutations in *P{UAS-kek1-gfp}*, we first generated suppressor stocks lacking *P{GAL4-ninaE.GMR}* by recombination. *P{UAS-kek1-gfp}* was then mobilized to either the *CyO* or the third chromosome using w^+*; Sp/CyO; Sb*Δ*2-3/Tm6,Hu* males as a source of transposase. Several lines of transposed P inserts were generated for every suppressor and tested by *P{GAL4-ninaE.GMR}* and *P{GawB}CY2* to ensure that the suppression phenotypes mapped to the P insert.

Molecular analysis: Genomic DNA was isolated from balanced stocks with QIAGEN (Valencia, CA) DNeasy columns according to the manufacturer's instructions. *kek1* alleles were selectively amplified from the P insert by PCR, utilizing primers specific to the *UAS* region and to the *gfp* fusion. A 3.5-kb fragment encoding the entire *kek1* transgene, 500 bases of upstream region and 100 bases of *gfp*, was sequenced in its entirety using cycle sequencing according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). To introduce the point mutations into a *pUAST-gfp* vector, we utilized the 3.5-kb *kek1-gfp* PCR fragment amplified from the alleles as a template. New primers encoding the 5′ and 3′ termini of *kek1* were flanked with AttB1 and AttB2 sites for subcloning into a *pUAST-gfp* vector via the Gateway system (Invitrogen, Carlsbad, CA). The presence of point mutations in the final clones was verified by sequencing.

Eye and chorion preparations: For chorion preparations, flies were raised at 25° and eggs were collected, washed, and cleared in lacto-Hoyer's solution for 48 hr at 60°. Images were captured under dark field on a Zeiss Axiophot microscope. Ovaries from females expressing GFP-tagged molecules were dissected in PBS and fixed for 10 min in 3.7% formaldehyde/PBS, washed three times in PBT (0.1% Tween-20), and brought to volume in 70% glycerol with SloFade (Molecular Probes, Eugene, OR). All fluorescent images were captured with a Leica TCS SP confocal microscope. For scanning electron micrographs (SEMs) of the adult eye, males were dehydrated in an increasing ethanol:dH_2O series, as described in Tio *et al.* (1994).

Antibody stainings: Anti-EGFR stainings on ovaries were performed as described in Peifer *et al.* (1993). Anti-EGFR was used at 1:5000. FITC-conjugated goat anti-rabbit secondary (Jackson Immunoresearch, West Grove, PA) was utilized at 1:300.

Cell culture and co-immunoprecipitations: Drosophila S3 cells were grown and maintained as described in Cherbas and Cherbas (1998). Cells were grown to a density of ~5 × 10^6 cells/ml and transfected by electroporation. Cells were cotransfected with 5 μg of *metallothionein-GAL4* (*mt-GAL4*) plasmid (Klueg *et al.* 2002), a copper-inducible GAL4 driver, and 5 μg of responder DNA and induced with 1 mM $CuSO_4$ for 22 hr. Cells were collected and gently pelleted by centrifugation at 2000 rpm and subsequently lysed in 1 ml of ice-cold Fehon buffer (Fehon *et al.* 1990) containing 1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 1 μM pepstatin A, and 0.3 μM aprotinin. Lysed cells were cleared by centrifugation at 14,000 rpm for 5 min at 4°. Supernatant was brought up to 5 ml in buffer, and antigen was immunoprecipitated with 0.5 μl of rabbit anti-GFP (CLONTECH, Palo Alto, CA). Samples were rotated for 2 hr at 4° and subsequently incubated with 150 μl of a 1:5 slurry of protein A Sepharose beads (Amersham-Pharmacia, Piscataway, NJ) in Fehon buffer for 1 hr at 4°. Beads were collected by gentle centrifugation (3000 rpm for 2 min at 4°) and washed five times in Fehon buffer. The last two washes were performed in Fehon buffer lacking detergent. Samples were resuspended in 12 μl of 2× Laemmli buffer, boiled for 5 min, and loaded in 8% polyacrylamide gels. Transfer to nitrocellulose membranes (Amersham-Pharmacia) was followed by Ponceau staining and subsequently blocked for 1 hr at room temperature (RT) in 5% nonfat dry milk (NFDM) with TBST (100 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween-20). Membranes were washed twice in TBST and then incubated with primary antisera at the following concentrations:

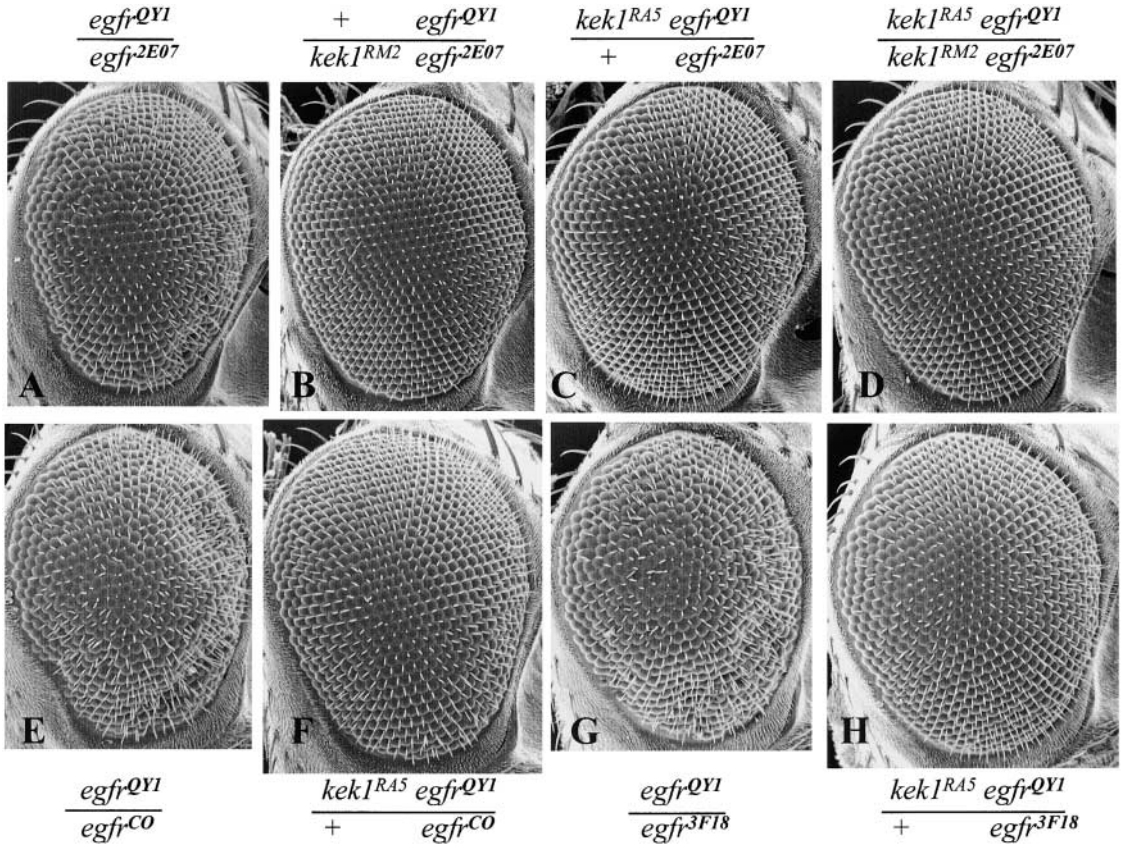

Figure 1.—Kek1 inhibits EGFR in the developing eye. (A, E, and G) Partial *egfr* loss of function was generated through *trans*-heterozygous combinations of alleles, resulting in eye roughening. (A) $egfr^{QY1}/egfr^{2E07}$; (E) $egfr^{QY1}/egfr^{CO}$; (G) $egfr^{QY1}/egfr^{3F18}$. Removal of one (B, C, F, and H) or both (D) copies of *kek1* suppresses the EGFR loss-of-function phenotypes to nearly wild type. (B) $egfr^{QY1}/kek1^{RM2}$, $egfr^{2E07}$; (C) $kek1^{RA5}$, $egfr^{QY1}/egfr^{2E07}$; (F) $kek1^{RA5}$, $egfr^{QY1}/egfr^{CO}$; (H) $kek1^{RA5}$, $egfr^{QY1}/egfr^{3F18}$; (D) $kek1^{RA5}$, $egfr^{QY1}/kek1^{RM2}$, $egfr^{2E07}$.

rabbit anti-EGFR (courtesy of Nick Baker; Lesokhin *et al.* 1999) at 1:5000 (2% NFDM in TBST), monoclonal anti-GFP (CLONTECH) at 1:1000 in TBST, and monoclonal anti-V5 (Invitrogen) at 1:1000 (1% NFDM in TBST). Incubations were done overnight at 4°, followed by five washes in TBST. Secondary HRP-conjugated goat-anti-rabbit or mouse antibody incubations (Jackson Immunoresearch) were done at 1:20,000 in 5% NFDM for 1 hr at RT, followed by five washes in TBST. Detection was performed by chemiluminescence (West Pico, Pierce, Rockford, IL), according to the manufacturer's instructions, utilizing Kodak Biomax MR-1 autoradiography film. Stripping and reprobing was performed according to the manufacturer's instructions.

RESULTS

Kek1 inhibits EGFR signaling in the eye: EGFR signaling is utilized reiteratively throughout eye development to mediate a multitude of cellular decisions, such as specification, proliferation, differentiation, and survival (Xu and Rubin 1993; Freeman 1996; Kumar *et al.* 1998; Kumar and Moses 2001). Although *kek1* is expressed in the eye imaginal disc, whether or not Kek1 functions to attenuate EGFR signaling during eye development has been unclear (Musacchio and Perrimon 1996). Previous studies using deficiencies that uncover the *kek1* locus reported that the null condition for *kek1* displayed no overt adult phenotypes (Musacchio and Perrimon 1996). Subsequently, however, subtle phenotypic effects of a *kek1* null were identified in oogenesis and demonstrated to suppress phenotypes resulting from reductions in EGFR signaling (Ghiglione *et al.* 1999). To determine whether Kek1 functions in a similar manner in the eye, we used ommatidial phenotypes to assay the ability of reduced *kek1* function to compensate for reductions in EGFR activity. Viable hypomorphic combinations of *egfr* that disrupt eye development were generated and compared to similar genotypes also lacking *kek1* function (Figure 1). Using *kek1* null mutations we removed one (Figure 1, B, C, F, and H) or two (Figure 1D) copies of *kek1* from these hypomorphic receptor backgrounds. In all cases, reduction or elimination of *kek1* activity resulted in strong suppression of these hypomorphic $egfr^{-}$ eye phenotypes. Thus, as in oogenesis, Kek1 appears to inhibit EGFR signaling during eye development, thereby validating the eye as a system in which to study the role of Kek1 in EGFR signaling.

Isolation of suppressors of Kek1 misexpression in the eye: Within Drosophila, Kek1 is one of six Kekkon family members, all of which share a similar extracellular structure of seven LRRs and a single Ig domain. In contrast to this common structure, misexpression exper-

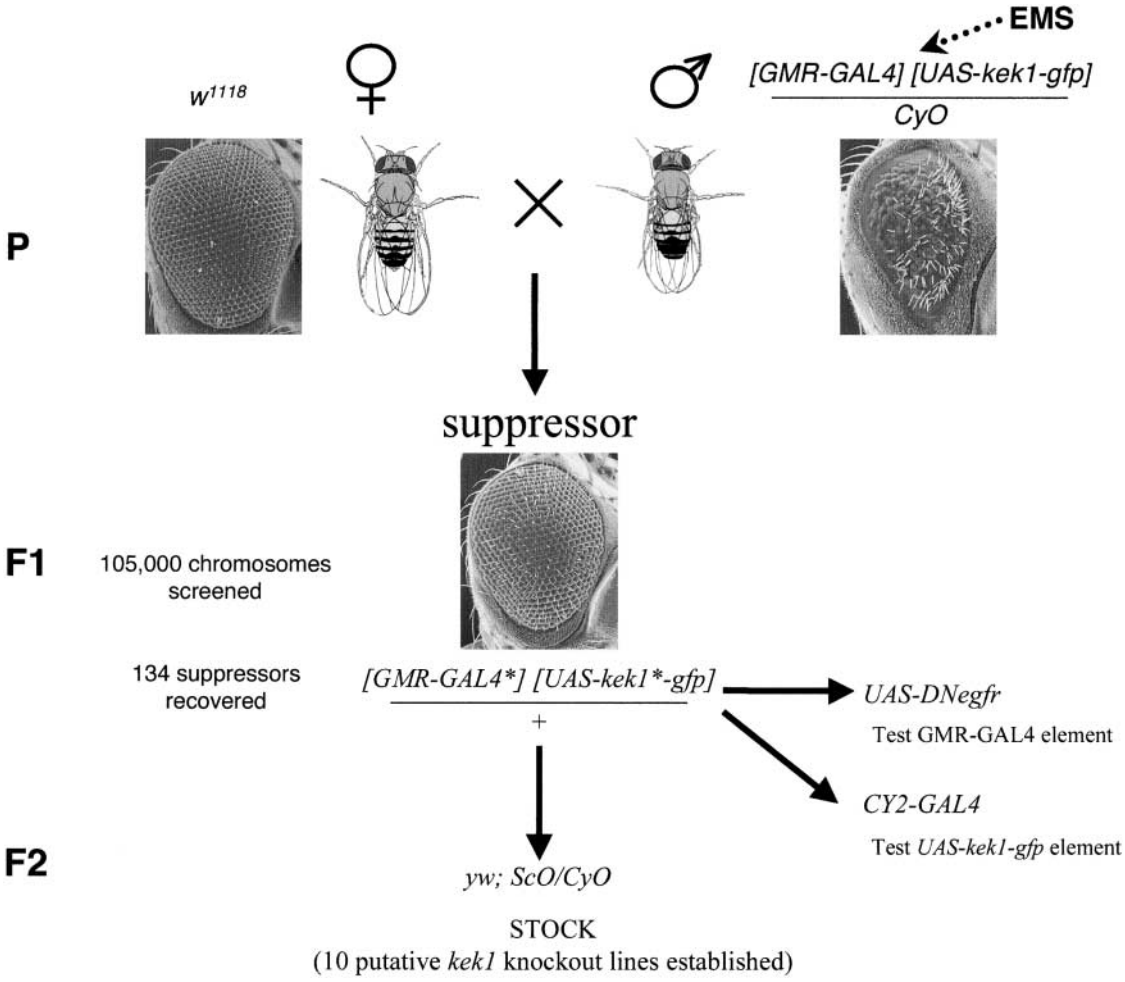

Figure 2.—Identification of suppressors of a *kek1-gfp* misexpression phenotype. *GMR-GAL4, UAS-kek1-gfp/CyO* males were mutagenized and crossed to *w; iso2; iso3* females. A total of 105,000 F_1 males and females were screened for suppression of the eye phenotype. Suppressors were crossed to a second chromosome balancer stock (*yw; ScO/CyO*) and balanced over *CyO* in the F_2 generation. F_2 stocks that maintained suppression were crossed to both a dominant negative form of EGFR (*UAS-DNegfr*) and a follicle cell driver (*CY2-GAL4*). Flies that display a severe rough eye due to DNEGFR misexpression are indicative of a fully functional *GMR-GAL4* driver. Flies that display suppression of strong chorion ventralization, as observed with misexpression of Kek1-GFP in follicle cells, are indicative of a loss of function in the *UAS-kek1-gfp* P insert. Missense mutations in *kek1-gfp* were preliminarily identified by GFP fluorescence in follicle cells, which denoted that the *kek1-gfp* transgene was expressed and translated appropriately and that no premature stop codons were induced by mutagenesis. Ten suppressor stocks were demonstrated to contain full driver function and a decrease in responder strength.

iments indicate that not all Kek family members have the ability to inhibit EGFR activity, suggesting that this trait is likely to be unique to Kek1 (Alvarado *et al.* 2004). This inhibitory function has been mapped to the extracellular and transmembrane region of Kek1, but the specific sequences responsible have yet to be identified (Ghiglione *et al.* 1999). To define the Kek1 elements responsible for this activity and to gain insight into the functional features that distinguish Kek1 from other family members, we took advantage of Kek1's inhibitory role during eye development. Due to the subtle nature of endogenous Kek1 function in the eye, *GMR-GAL4* was used to drive expression of *UAS-kek1-gfp* specifically in the eye-antennal disc. This directed misexpression of Kek1 in the developing eye resulted in a severe rough eye phenotype in adults similar to that observed with misexpression of dominant negative versions of EGFR and consistent with Kek1's ability to inhibit EGFR signaling (Figures 2 and 3; Freeman 1996).

Using this Kek1-dependent eye phenotype we designed a GFP-based genetic screen to recover LOF missense mutations in the *UAS-kek1-gfp* transgene (Figure 2). Flies misexpressing Kek1-GFP (*GMR-GAL4, UAS-kek1-gfp*) were mutagenized and outcrossed, and the F_1 were screened for suppressors of the rough eye phenotype. Mutations affecting *GMR-GAL4* function were identified by crossing all F_1 suppressors to a *UAS*-dominant negative *egfr (DNegfr)* line. Suppressor lines that failed to produce a phenotype in combination with dominant

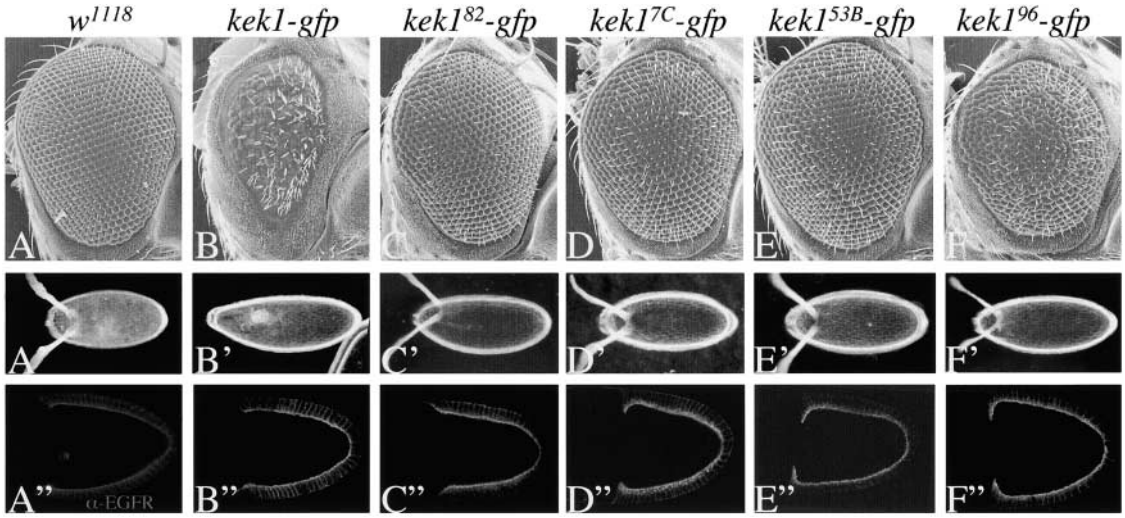

FIGURE 3.—Class I suppressors disrupt Kek1 function but retain wild-type localization. (A–F) SEMs of wild-type eyes (A) *vs.* class I suppressors of *GMR-GAL4, UAS-kek1-gfp* (B–F). (A′–F′) Comparison of wild-type chorions (A′) *vs.* class I suppressors of *CY2-GAL4, UAS-kek1-gfp* (B′–F′). (A″–F″) EGFR is localized apical-laterally in wild-type follicle cells (A″), similar to Kek1-GFP and class I mutants (B″–F″).

negative epidermal growth factor receptor (DNEGFR) were assumed to represent GAL4 mutations and were not pursued further. Since *kek1* missense alleles were likely to be most informative, the C-terminal GFP tag was then used as a marker to distinguish missense mutations from those that eliminated Kek1 expression (*e.g.*, nonsense and frameshift mutations in *kek1* or *GAL4* alleles).

For each of the nine remaining lines, as well as one GFP negative line (representing a putative nonsense/frameshift mutation), the *kek1-gfp* transgene was recombined away from the *GMR-GAL4* insert, retested for suppression with *GMR-GAL4*, and mobilized to different chromosomes. New inserts for a given suppressor were tested again with *GMR-GAL4*. In all cases, suppression segregated with the *UAS-kek1-gfp* transgene consistent with intragenic mutations. Thus, from ~105,000 haploid genomes screened, 10 suppressors that were functionally consistent with knockouts of *kek1* (*kek1*^{kok}) were recovered. These lines were then subjected to further analysis as described below.

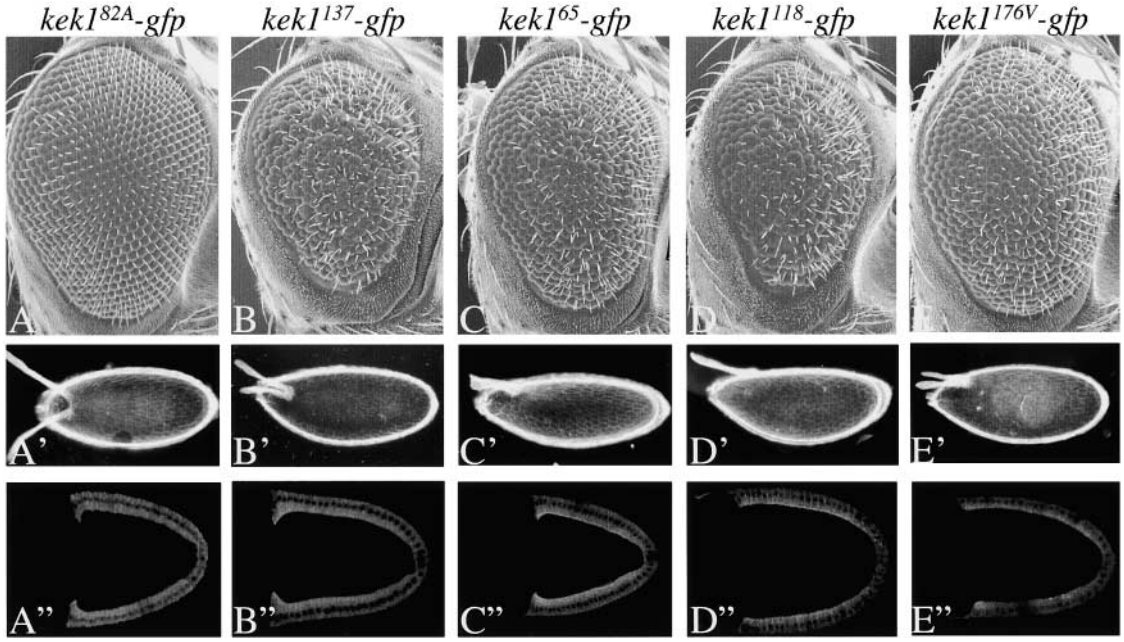

FIGURE 4.—Class II suppressors disrupt Kek1 function and localization. (A–E). SEMs of class II suppressors of *GMR-GAL4, UAS-kek1-gfp.* (A′–E′) Comparison of class II suppressors of *CY2-GAL4, UAS-kek1-gfp.* (A″–E″) Partial or complete loss of apical subcellular distribution is evident in these suppressors.

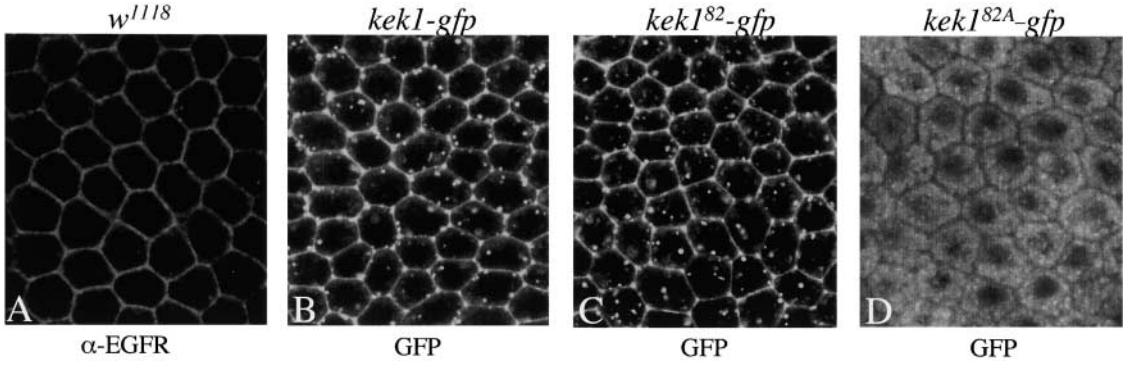

FIGURE 5.—Class II suppressors display aberrant Kek1 localization. Cross section of follicle cells from stage 10 egg chambers. (A) Anti-EGFR staining in a wild-type egg chambers. (B–D) GFP fluorescence pattern by misexpression of *kek1-gfp* (B), class I mutant *kek1^{82}-gfp* (C), and class II mutant *kek1^{82A}-gfp* (D) with *CY2-GAL4*. A–C exhibit clear membrane localization, in contrast to D, which localizes in a complementary pattern.

***kek1^{kok}* alleles represent two distinct classes:** The 10 suppressors range from partial to complete, suggesting that they represent an allelic LOF series for *kek1* (Figures 3 and 4). In addition, given the assay used to recover these alleles, it is possible that some alleles could be eye specific. To address this question, all *kek1^{kok}* lines were crossed to *P{GawB}CY2* (*CY2-GAL4*), which allowed their activity to be examined in a second tissue, the follicular epithelium. *CY2-GAL4*-directed misexpression of wild-type Kek1-GFP inhibits EGFR in the follicle cells, resulting in strong ventralization of the chorion (Figure 3). In contrast, misexpression of all *kek1^{kok}* lines with *CY2-GAL4* displayed strong LOF effects, consistent with disruption of the activity of the *kek1* transgene. The effects ranged from weakly ventralized to wild-type chorions, indicative of minimal to no Kek1 activity, respectively. The LOF effect of each putative *kek1^{kok}* allele was comparable in oogenesis and the adult eye, indicating that no eye-specific alleles were recovered. This is in agreement with the notion that Kek1 inhibits EGFR in multiple tissues via the same mechanism.

Using the *CY2-GAL4* driver, the 10 suppressor lines were also assayed for GFP expression in follicle cells to determine if they represented putative missense or nonsense/frameshift mutations. Misexpression of wild-type Kek1-GFP displayed apical localization in the follicle cells of stage 10 egg chambers, similar to that observed for endogenous EGFR (Figure 3). Nine suppressor lines exhibited GFP expression, while 1 line exhibited no GFP expression. Strikingly, analysis of the GFP expression pattern indicated that the *kek1^{kok}* alleles could be grouped into two distinct classes on the basis of their subcellular distribution. Class I alleles (*kek1^{53B}*, *kek1^{7C}*, *kek1^{82}*, and *kek1^{96}*) localized predominantly to the apical-lateral membrane in the same manner as wild-type Kek1-GFP (Figure 3). In contrast, class II alleles (*kek1^{82A}*, *kek1^{118}*, *kek1^{65}*, *kek1^{137}*, and *kek1^{176V}*) displayed aberrant localization. In these lines the distribution of Kek1-GFP appeared primarily cytoplasmic, displaying a reduced bias for the apical region of follicle cells (Figures 4 and 5). Finally, class II lines exhibiting strong suppression generally displayed higher degrees of mislocalization than did lines displaying moderate suppression.

Knockouts of *kek1* represent missense mutations in the extracellular region: On the basis of the data above, all 10 suppressors appeared to represent mutations in the *kek1* transgene that disrupt its ability to inhibit EGFR. To confirm this, the *kek1-gfp* transgene was sequenced from each line, including *kek^{191}*, the putative nonsense/frameshift allele. Changes in the *kek1-gfp* transgene sequence were identified in all cases and were found to map throughout the extracellular domain, consistent with previous observations documenting the importance of this portion of Kek1. Specifically, mutations were identified in the first, second, and third LRRs, the C-terminal cysteine-rich flank (C-flank), and the Ig domain (Figure 6A). No mutations in the signal sequence, transmembrane region, or cytoplasmic domain were recovered. As described above, the mutations were grouped into two classes according to GFP distribution pattern (Table 1). The four class I suppressors represented changes in only two positions. Of particular interest, mutations in glycine 160, located in the second LRR, were independently isolated three times. Two of these alleles, *kek1^{53B}* and *kek1^{82}*, encode a change to serine (G160S), whereas in the third isolate, *kek1^{7C}*, this residue has been converted to aspartic acid (G160D). All three of these alleles result in almost complete suppression. In contrast, only partial suppression is observed in the last class I allele, *kek1^{96}*, which is the result of a leucine-to-phenylalanine (L136F) change in the first LRR.

The five class II suppressors represent mutations in four different positions. Surprisingly, all of these alleles, which disrupt Kek1 localization, are changes in proline residues. The partial suppressor *kek1^{137}* encodes a P187S change in the third LRR. Proline 309, located in the C-flank, is mutant in two suppressors; *kek1^{118}* encodes a change to serine (P309S), whereas in *kek1^{65}* P309 is changed to leucine (P309L). *kek1^{82A}* (P329S), the strongest suppressor identified, and *kek1^{176V}* (P356S), a moder-

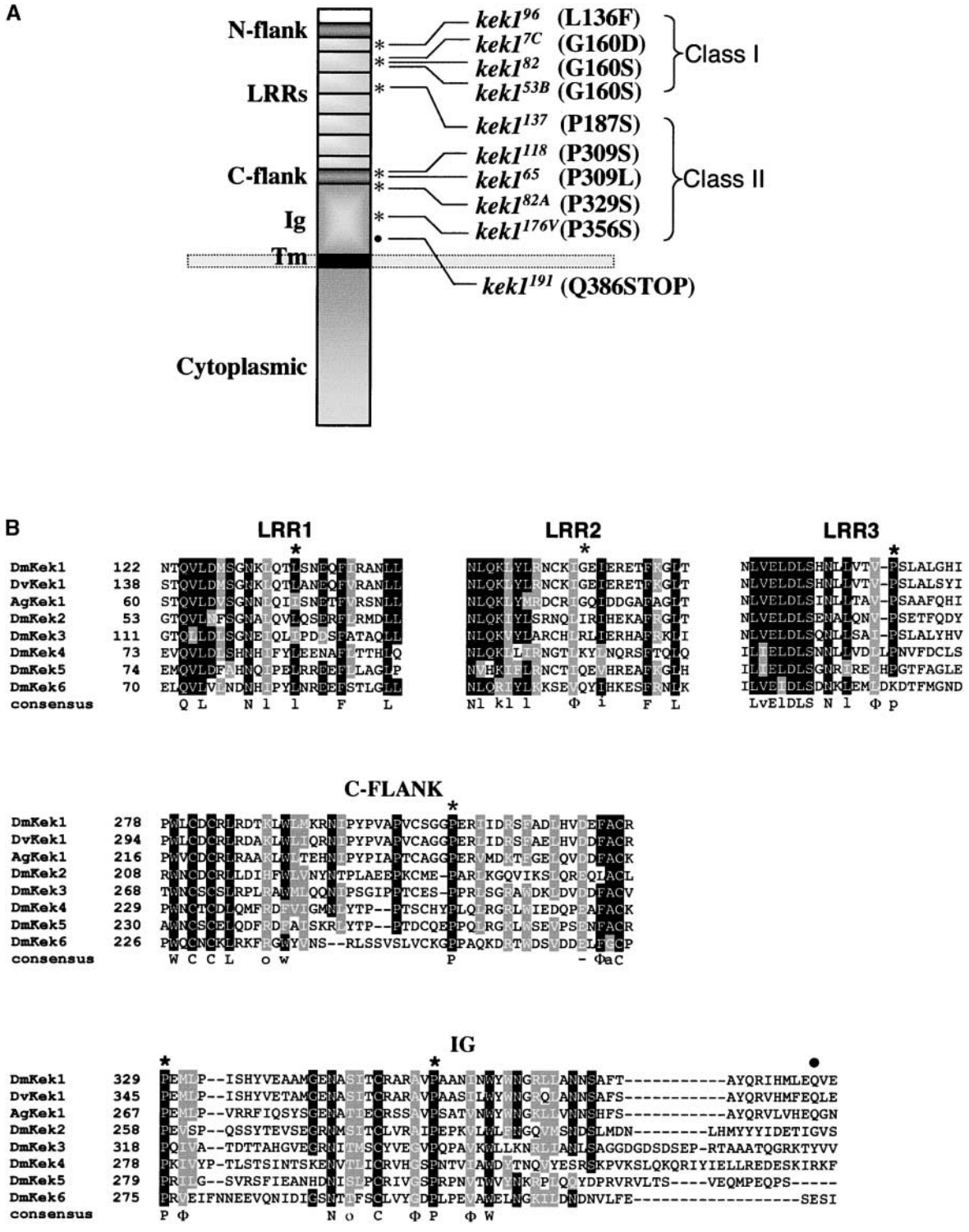

FIGURE 6.—Kek1 mutations are distributed throughout the extracellular region. (A) Kek1 is a single-pass transmembrane domain containing seven LRRs and a single Ig domain in the extracellular region. The LRRs are flanked by N- and C-terminal cysteine-rich flanks (N- and C-flank). Four class I alleles cluster to the LRRs (*kek1*^{96}, *kek1*^{7C}, *kek1*^{82}, and *kek1*^{53B}), accounting for changes in only two amino acids (L136 and G160), whereas class II allele *kek1*^{137} encodes a P187S change in the third LRR. All other mutants distal to the LRRs are class II alleles: *kek1*^{118} (P309S) and *kek1*^{65} (P309L) in the C-flank and *kek1*^{82A} (P329) and *kek1*^{176V} in the Ig domain (P356). The nonsense mutant *kek1*^{191} (Q386*) in the Ig domain was originally identified on the basis of the lack of GFP fluorescence. (B) Protein sequence alignment of the first three LRRs, C-flank, and Ig domains of all Kek family members and Kek1 orthologs in *D. virilis* and *A. gambiae*. All class I missense mutations occurred in residues conserved in all members and orthologs with the exception of G160, which is found only in DvKek1 and AgKek1.

TABLE 1

Data summary for missense suppressors of Kek1-GFP misexpression in the eye and ovary

	GMR-GAL4, UAS-kek1-gfp	Change	Eye (stock)	Chorion (*CY2-GAL4*)	GFP localization
Class I	*kek1-gfp*	Control	R3	V3	Apical
	kek1^{96}-gfp	L136F (CTC-TTC)	R1	V1–V2	Apical
	kek1^{7C}-gfp	G160D (GGC-GAG)	wt	wt	Apical
	kek1^{53B}-gfp	G160S (GGC-AGC)	wt	wt	Apical
	kek1^{82}-gfp	G160S (GGC-AGC)	wt	wt	Apical
Class II	*kek1^{137}-gfp*	P187S (CCC-TCC)	R2	V1	Mislocalized
	kek1^{65}-gfp	P309L (CCC-CTC)	R2	V2	Mislocalized
	kek1^{118}-gfp	P309S (CCC-TCC)	R2	V1–V2	Mislocalized
	kek1^{82A}-gfp	P329S (CCG-TCG)	wt	wt	Mislocalized
	kek1^{176V}-gfp	P356S (CCA-TCA)	R1	V1–V2	Mislocalized

Suppressors separated according to classes. Amino acid changes accounting for the suppression phenotype are shown. Suppressor stocks were maintained as recombinants with the *GMR-GAL4* driver (stock), which were crossed to *CY2-GAL4* to test suppression in the ovary. Eye phenotypes are classified as a range between mild roughness (R1) and severe roughness with a decrease in eye size (R4). Likewise, chorion phenotypes are classified according to the degree of ventralization, ranging from weak (V1) to complete ventralization (V4). GFP localization was followed in somatic follicle cells. Normal apical localization is observed with the control Kek1-GFP protein and class I suppressors, whereas class II suppressors display abnormal cellular distribution (mislocalized).

ate suppressor, are both located in the Ig domain. Finally, to validate the use of GFP fluorescence as a tool to discern missense from nonsense/frameshift mutations, we examined the nature of the lesion in *kek1^{191}*, a GFP-negative suppressor. In agreement with our prediction, sequencing of *kek1^{191}* revealed the introduction of a premature stop codon (Q386*), which truncates the protein within the Ig domain.

All members of the Kek family display significant sequence similarity throughout their extracellular regions. Comparison of the positions of the *kek^{kok}* mutations across family members and Kek1 orthologs reveals that only G160 is unique to Kek1, supporting a crucial role for this residue in Kek1 inhibition of EGFR (Figure 6B). All other positions contain residues that are conserved among other family members, suggesting that these latter residues act in a permissive, rather than instructive, fashion in EGFR inhibition by Kek1.

The effects of class I and class II alleles are mediated through distinct mechanisms: Because of the localization patterns of Kek1-GFP in class I *vs.* class II alleles, we explored the possibility that loss of EGFR inhibition in the two classes occurred via different mechanisms. Both EGFR and class I mutants localize apically, while class II mutants localize primarily within the cytoplasm. Therefore, class I suppressors might directly affect the affinity of Kek1 for the EGFR, while class II alleles might indirectly affect inhibition by preventing colocalization of Kek1 with the receptor. Point mutations corresponding to class I and II alleles were introduced into the *pUAST-gfp* vector and tested for their ability to interact with EGFR by co-immunoprecipitation from S3 cells (Figure 7; data not shown). Class I alleles displayed a reduction in EGFR binding proportional to their phenotypic strength, while class II alleles displayed normal EGFR binding. These results are consistent with the hypothesis that class I alleles directly disrupt EGFR binding, while class II alleles affect subcellular distribution since their affinity for EGFR does not appear to be otherwise compromised.

Last, mutations in G160 were independently isolated three times, suggesting that this residue is a critical determinant of Kek1's interaction with EGFR. Wild-type Kek1, in addition to its ability to associate with the EGFR, is also capable of associating with itself (Figure 7B). The mutation G160S, found in the alleles *kek1^{53B}* and *kek1^{82}*, exhibits reduced binding to EGFR, but retains wild-type activity when tested for the ability to associate with Kek1. This argues that G160S is unlikely to disrupt the overall structure of Kek1, but rather supports the notion that G160 functions in a specific manner to facilitate the interaction between Kek1 and EGFR (Figure 7B).

DISCUSSION

Here we identify and characterize sequence elements in Kek1 that mediate its role in EGFR signaling. Nine missense alleles were identified in *kek1* and shown to fall into two classes that disrupt normal protein function via distinct mechanisms. Consistent with the previously ascribed importance of the extracellular domain, all LOF mutations were located in the extracellular domain of the protein, mapping to the LRRs, C-flank, and Ig domain.

Kek1 is a general inhibitor of EGFR signaling: The role of Kek1 as a negative regulator of EGFR signaling

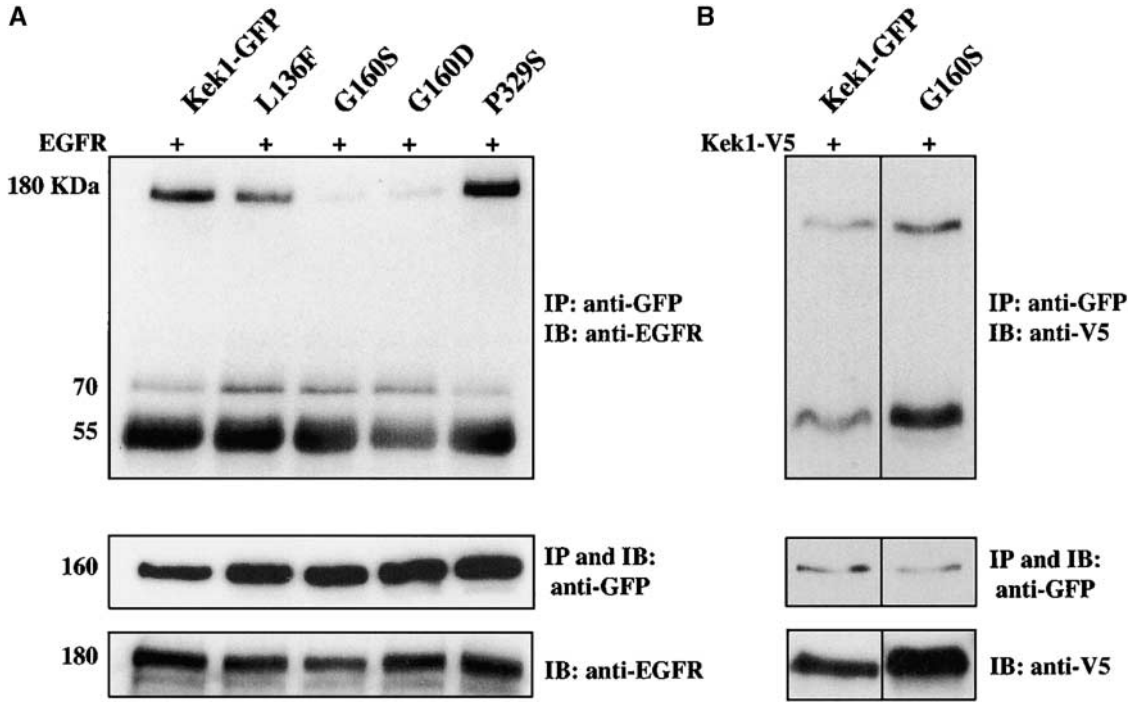

Figure 7.—Class I alleles affect Kek1's affinity for EGFR. (A) GFP-tagged forms of Kek1 and four alleles were immunoprecipitated (IP) from S3 cells and immunoblotted (IB) with anti-EGFR (top). Filters were stripped and reprobed with anti-GFP (middle) to control for Kek1-GFP protein levels. A small sample of cells was lysed and directly immunoblotted with anti-EGFR (bottom) to control for the presence of equal EGFR amounts in all samples. An L136F change in Kek1 results in a moderate reduction of EGFR binding, whereas alleles encoding changes in G160 display greatly decreased affinities for EGFR. These reductions in affinity are in agreement with the variable phenotypic strength of each suppressor. In contrast, the class II mutant P329S (*kek1*^{82A}-*gfp*) does not affect Kek1 binding to EGFR. (B) G160 does not affect Kek1 homodimerization. GFP-tagged Kek1 and Kek1^{82} (G160) were immunoprecipitated and immunoblotted with anti-V5 (top) to assay for the presence of Kek1-V5 in the complex. Protein expression levels were controlled for by stripping and reprobing the precipitate with anti-GFP (middle) and by immunoblotting cell lysates with anti-V5 (bottom).

had been reported in oogenesis and it was unclear whether this effect was tissue or ligand specific. We demonstrate by LOF and GOF experiments that Kek1 inhibits EGFR signaling in the developing eye. Moreover, given the lack of an overt *kek1* LOF phenotype in the eye, it was surprising to discover that hemizygosity for *kek1* rescued EGFR hypomorphic phenotypes. Along with the previous demonstration that Kek1 functions in oogenesis, our data indicate that Kek1 functions in a dose-dependent manner to attenuate EGFR signaling in multiple tissues (Ghiglione *et al.* 1999). It will be interesting to determine if this inhibitory function underlies the conservation of Kek1 over 250 million years from Drosophila to Anopheles (Derheimer *et al.* 2004, accompanying article).

Class I alleles define EGFR-binding specificity: Kek family members contain a similar extracellular structure, consisting of LRRs flanked by cysteine-rich motifs, followed by a single Ig domain. In spite of this common structure, inhibition of EGFR signaling appears unique to Kek1 (Alvarado *et al.* 2004). To define the sequence elements that attribute this function to Kek1, we identified knockouts of a Kek1 misexpression phenotype in the eye. Two classes of alleles, each displaying a distinct pattern of subcellular localization, were recovered. Class I alleles exhibit wild-type localization and changes map to the first and second LRRs of Kek1. LRRs are generally involved in protein-protein interactions and are found in a multitude of proteins and organisms, ranging from bacteria to vertebrates. Typical LRRs consist of stretches of 21–25 amino acids and are defined by repeats of the conserved sequence LxxLxLxxN/CxL, where conservative substitutions of leucine for similar hydrophobic residues are common. However, the rest of the repeat can be highly divergent. Structurally, these motifs are composed of a β-sheet, defined by the conserved sequence, which is connected to an α-helix. The entire set of LRRs is thought to form a horseshoe structure, with the hydrophobic β-sheets lining the inside of the structure and the α-helices exposed to the outer surface (Kobe and Deisenhofer 1994, 1995; Kobe and Kajava 2001).

The class I allele *kek1*^{96} disrupts a conserved leucine in the first LRR and is a partial suppressor. L136 is conserved in all Kek family members, suggesting that it does not directly dictate EGFR-binding specificity. In addition, the amino acids surrounding L136 at the $n - 2$, $n - 1$, and $n + 1$ positions are highly conserved in all Kek proteins. This reinforces the notion that L136

plays a structural rather than a direct role in association with the receptor. Consistent with a partial loss of activity *in vivo*, L136F also displays a reduction in EGFR-binding affinity as shown by co-immunoprecipitation. This partial loss-of-function phenotype could be due to the fairly conservative nature of the substitution, where leucine is changed to phenylalanine, a bulkier hydrophobic residue. Alternatively, the first LRR may meet only a small structural requirement for Kek1 function.

The three remaining class I alleles change G160 in the second LRR and may represent the most functionally relevant mutations uncovered in our screen. Several lines of evidence support this suggestion. First, glycine 160 is mutated in three separate suppressors: to serine in *kek1*^{82} and *kek1*^{53B} and to aspartic acid in *kek1*^{7C}. Second, these alleles display strong suppression of the Kek1-GFP misexpression phenotype, in both the eye and the ovary, while exhibiting correct subcellular localization. Third, G160 is conserved in Kek1 orthologs from *Drosophila virilis* and *Anopheles gambiae*, but is divergent in the other Kek family members. Finally, these changes reduce the affinity of Kek1 for EGFR, but not for itself in co-immunoprecipitation experiments. These lines of evidence demonstrate that G160 is likely to play an instructive rather than a permissive role in mediating EGFR binding and inhibition. Together, the data from class I alleles suggest that the first and second LRRs function together to direct EGFR binding, consistent with recent findings that the LRRs are essential for inhibition of EGFR (Ghiglione *et al.* 2003). The first repeat consequently may be required for the correct positioning of the second repeat in which G160 specifies EGFR binding.

Class II alleles affect Kek1 subcellular distribution: Notably, all class II alleles alter Kek1 subcellular localization and involve changes in proline residues that are conserved, with one exception, throughout the Kek family. Whereas EGFR, Kek1-GFP, and class I mutants localize primarily to the apical membrane of polarized follicle cells, class II alleles localize more uniformly throughout the cell and appear cytoplasmic in their distribution. Furthermore, within class II, strong suppressors display higher degrees of mislocalization than do intermediate suppressors. The class II allele *kek1*^{137} (P187S) affects the third LRR and is a suppressor with intermediate activity. This proline is conserved in all Kek1 orthologs and Kek family members, with the exception of Kek6. Two alleles of moderate strength, *kek1*^{65} and *kek1*^{118}, both mapped to a single residue (P309) located in the C-flank. N-terminal and C-terminal cysteine-rich flanks are capping motifs commonly associated with LRRs and are defined by the conserved positioning of cysteine residues. All Kek family members contain a proline at the same relative position as P309. *kek1*^{65} (P309L) behaves as a slightly stronger suppressor than *kek1*^{118} (P309S), consistent with the higher degree of subcellular mislocalization in *kek1*^{65}. This minor difference in protein localization is likely caused by the nature of the substituting amino acid. The two remaining class II alleles are mutations in the Ig domain. Proteins with Ig domains constitute a superfamily of molecules with varied function in which the Ig domain confers protein-binding properties. *kek1*^{82A} (P329S) is the result of a change in the first amino acid of the Ig domain, which is conserved in all Kek family members. The allele with this change was the strongest suppressor identified in the screen and localizes uniformly throughout the cell. The mutation encoded by *kek1*^{176V} (P356S) is also within the Ig domain, but it represents a moderate suppressor. Consistent with only a partial LOF in Kek1, this mutant protein, although localized abnormally, displayed a slight bias for the apical surface of follicle cells. Finally, co-immunoprecipitation experiments between class II alleles and EGFR reveals that most class II alleles have the intrinsic ability to bind the receptor with wild-type affinity (Figure 7; data not shown). This strongly suggests that the suppression observed *in vivo* is due to reduced apical membrane localization of Kek1, consequently limiting its ability to interact with and inhibit the receptor. Thus, class II alleles define a set of distinct proline residues that promote Kek1 function through effects on subcellular localization.

Role of the cytoplasmic domain: The cytoplasmic region of Kek1 was previously reported to be dispensable for EGFR binding and inhibition (Ghiglione *et al.* 1999). In agreement with these findings, no mutations affecting Kek1 function were recovered in this region. Interestingly, however, the C-terminal tail (48 amino acids) represents the most highly conserved portion of Kek1 between Drosophila and Anopheles (Derheimer *et al.* 2004, accompanying article). This portion of Kek1, like Kek2 and Kek5, contains a putative type 1 binding site (S/T-X-I/V/G) for proteins containing PDZ domains. PDZ proteins often play a role in trafficking or scaffolding of membrane-associated proteins (Harris and Lim 2001). Supporting this deletion of the cytoplasmic domain of Kek1 can disrupt its trafficking (Ghiglione *et al.* 2003). However, the fact that no mutations in the cytoplasmic domain were recovered in our screen suggests that loss of the cytoplasmic domain does not compromise Kek1 function in the eye. This is consistent with our unpublished observations and suggests that this region may contribute to Kek1's inhibitory function in a more refined or tissue-specific fashion. Alternatively, the basis for this conservation might lie in an EGFR-independent role.

Conclusions: Inhibition of EGFR signaling by the Kek family member, Kek1, occurs in multiple developmental processes and is mediated by the extracellular portion of Kek1. Mutations affecting Kek1's inhibitory activity are spread throughout the extracellular region, but reflect different LOF mechanisms. Specificity for EGFR binding is likely to reside to a large degree in the second LRR at G160. This residue was mutated in three different suppressors, affects the affinity of Kek1 for EGFR,

and is unique to Kek1 among Kek family members. On the basis of this finding we propose that the second LRR underlies the binding specificity of Kek1 for EGFR and therefore its inhibitory function. Given this and the plethora of secreted and transmembrane molecules containing LRRs within the Drosophila genome, it will be important to determine if this sequence represents an EGFR interaction motif present in additional LRR-containing molecules and to decipher their contributions to EGFR signaling. Likewise, it will be interesting to determine if the analogous region in other Kek family members directs their function and if they act in a related manner on distinct receptors.

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LITERATURE CITED

Alvarado, D., A. H. Rice and J. B. Duffy, 2004 Bipartite inhibition of Drosophila epidermal growth factor receptor by the extracellular and transmembrane domains of Kekkon1. Genetics (in press).

Ashburner, M., 1989 *Drosophila: A Laboratory Handbook.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Bai, J., W. Chiu, J. Wang, T. Tzeng, N. Perrimon *et al.*, 2001 The cell adhesion molecule Echinoid defines a new pathway that antagonizes the Drosophila EGF receptor signaling pathway. Development **128:** 591–601.

Cherbas, L., and P. Cherbas, 1998 Cell culture, pp. 319–346 in *Drosophila: A Practical Approach*, edited by D. B. Roberts. Oxford University Press, Oxford.

Derheimer, F. A., C. M. MacLaren, B. P. Weasner, D. Alvarado and J. B. Duffy, 2004 Conservation of an inhibitor of the epidermal growth factor receptor, Kekkon1, in Dipterans. Genetics **166:** 213–224.

Dominguez, M., J. D. Wasserman and M. Freeman, 1998 Multiple functions of the EGF receptor in Drosophila eye development. Curr. Biol. **8:** 1039–1048.

Fehon, R. G., P. J. Kooh, I. Rebay, C. L. Regan, T. Xu *et al.*, 1990 Molecular interactions between the protein products of the neurogenic loci Notch and Delta, two EGF-homologous genes in Drosophila. Cell **61:** 523–534.

Freeman, M., 1996 Reiterative use of the EGF receptor triggers differentiation of all cell types in the Drosophila eye. Cell **87:** 651–660.

Ghiglione, C., K. L. Carraway, III, L. T. Amundadottir, R. E. Boswell, N. Perrimon *et al.*, 1999 The transmembrane molecule kekkon 1 acts in a feedback loop to negatively regulate the activity of the Drosophila EGF receptor during oogenesis. Cell **96:** 847–856.

Ghiglione, C., L. Amundadottir, M. Andresdottir, D. Bilder, J. A. Diamonti *et al.*, 2003 Mechanism of inhibition of the Drosophila and mammalian EGF receptors by the transmembrane protein Kekkon 1. Development **130:** 4483–4493.

Harris, B. Z., and W. A. Lim, 2001 Mechanism and role of PDZ domains in signaling complex assembly. J. Cell Sci. **114:** 3219–3231.

Hime, G. R., M. P. Dhungat, A. Ng and D. D. Bowtell, 1997 D-Cbl, the Drosophila homologue of the c-Cbl proto-oncogene, interacts with the Drosophila EGF receptor in vivo, despite lacking C-terminal adaptor binding sites. Oncogene **14:** 2709–2719.

Klueg, K. M., D. Alvarado, M. A. Muskavitch and J. B. Duffy, 2002 Creation of a GAL4/UAS-coupled inducible gene expression system for use in Drosophila cultured cell lines. Genesis **34:** 119–122.

Kobe, B., and J. Deisenhofer, 1994 The leucine-rich repeat: a versatile binding motif. Trends Biochem. Sci. **19:** 415–421.

Kobe, B., and J. Deisenhofer, 1995 A structural basis of the interactions between leucine-rich repeats and protein ligands. Nature **374:** 183–186.

Kobe, B., and A. V. Kajava, 2001 The leucine-rich repeat as a protein recognition motif. Curr. Opin. Struct. Biol. **11:** 725–732.

Kumar, J. P., and K. Moses, 2001 EGF receptor and Notch signaling act upstream of Eyeless/Pax6 to control eye specification. Cell **104:** 687–697.

Kumar, J. P., M. Tio, F. Hsiung, S. Akopyan, L. Gabay *et al.*, 1998 Dissecting the roles of the Drosophila EGF receptor in eye development and MAP kinase activation. Development **125:** 3875–3885.

Lesokhin, A. M., S. Y. Yu, J. Katz and N. E. Baker, 1999 Several levels of EGF receptor signaling during photoreceptor specification in wild-type, Ellipse, and null mutant Drosophila. Dev. Biol. **205:** 129–144.

Musacchio, M., and N. Perrimon, 1996 The *Drosophila kekkon* genes: novel members of both the leucine-rich repeat and immunoglobulin superfamilies expressed in the CNS. Dev. Biol. **178:** 63–76.

Nilson, L. A., and T. Schupbach, 1999 EGF receptor signaling in *Drosophila* oogenesis. Curr. Top. Dev. Biol. **44:** 203–243.

Pai, L. M., G. Barcelo and T. Schupbach, 2000 D-cbl, a negative regulator of the Egfr pathway, is required for dorsoventral patterning in *Drosophila* oogenesis. Cell **103:** 51–61.

Peifer, M., S. Orsulic, D. Sweeton and E. Wieschaus, 1993 A role for the Drosophila segment polarity gene armadillo in cell adhesion and cytoskeletal integrity during oogenesis. Development **118:** 1191–1207.

Pruess, M., W. Fleischmann, A. Kanapin, Y. Karavidopoulou, P. Kersey *et al.*, 2003 The Proteome Analysis database: a tool for the in silico analysis of whole proteomes. Nucleic Acids Res. **31:** 414–417.

Queenan, A. M., A. Ghabrial and T. Schupbach, 1997 Ectopic activation of torpedo/Egfr, a Drosophila receptor tyrosine kinase, dorsalizes both the eggshell and the embryo. Development **124:** 3871–3880.

Schweitzer, R., and B. Z. Shilo, 1997 A thousand and one roles for the Drosophila EGF receptor. Trends Genet. **13:** 191–196.

Schweitzer, R., R. Howes, R. Smith, B.-Z. Shilo and M. Freeman, 1995 Inhibition of *Drosophila* EGF receptor activation by the secreted protein Argos. Nature **376:** 699–702.

Stevens, L., 1998 Twin peaks: Spitz and Argos star in patterning of the Drosophila egg. Cell **95:** 291–294.

Tio, M., C. Ma and K. Moses, 1994 spitz, a Drosophila homolog of transforming growth factor-alpha, is required in the founding photoreceptor cells of the compound eye facets. Mech. Dev. **48:** 13–23.

Van Buskirk, C., and T. Schupbach, 1999 Versatility in signalling: multiple responses to EGF receptor activation during Drosophila oogenesis. Trends Cell Biol. **9:** 1–4.

Wasserman, J. D., and M. Freeman, 1998 An autoregulatory cascade of EGF receptor signaling patterns the Drosophila egg. Cell **95:** 355–364.

Xu, T., and G. Rubin, 1993 Analysis of genetic mosaics in developing and adult Drosophila tissues. Development **117:** 1223–1237.

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