Development 130, 4483-4493

doi:10.1242/dev.00617

DEVELOPMENT AND DISEASE

Mechanism of inhibition of the *Drosophila* and mammalian EGF receptors by the transmembrane protein Kekkon 1

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Accepted 20 May 2003

SUMMARY

The transmembrane protein Kekkon 1 (Kek1) has previously been shown to act in a negative feedback loop to downregulate the *Drosophila* Epidermal Growth Factor Receptor (DER) during oogenesis. We show that this protein plays a similar role in other DER-mediated developmental processes. Structure-function analysis reveals that the extracellular Leucine-Rich Repeat (LRR) domains of Kek1 are critical for its function through direct association with DER, whereas its cytoplasmic domain is required for apical subcellular localization. In addition, the use of chimeric proteins between Kek1 extracellular and transmembrane domains fused to DER intracellular domain indicates that Kek1 forms an heterodimer with DER in vivo. To characterize more precisely the mechanism underlying the Kek1/DER interaction, we used mammalian ErbB/EGFR cell-based assays. We show that Kek1 is capable of physically interacting with each of the known members of the mammalian ErbB receptor family and that the Kek1/EGFR interaction inhibits growth factor binding, receptor autophosphorylation and Erk1/2 activation in response to EGF. Finally, in vivo experiments show that Kek1 expression potently suppresses the growth of mouse mammary tumor cells derived from aberrant ErbB receptors activation, but does not interfere with the growth of tumor cells derived from activated Ras. Our results underscore the possibility that Kek1 may be used experimentally to inhibit ErbB receptors and point to the possibility that, as yet uncharacterized, mammalian transmembrane LRR proteins might act as modulators of growth factor signalling.

Key words: *kek1*, EGFR, Negative feedback loop, LRR domains, Mammary tumor

INTRODUCTION

The four known members of the mammalian ErbB family of cell surface growth factor receptor tyrosine kinases (RTK), ErbB2, ErbB3, ErbB4 and EGFR, contribute to a variety of developmental processes (Gassmann et al., 1995; Lee et al., 1995; Miettinen et al., 1995; Threadgill et al., 1995; Erickson et al., 1997; Burden and Yarden, 1997). In addition, overexpression of these receptors has been observed in numerous human solid tumors (Menard et al., 2000; Aunoble et al., 2000), and it is widely believed that the aberrant activation of their protein tyrosine kinase activities actively contributes to tumor progression (Kim and Muller, 1999). Thus, much emphasis has been placed on understanding the biochemical mechanisms by which ErbB receptors are activated in tumor cells, and on the development of ErbB antagonists that could function as anti-cancer agents. Indeed, Herceptin, a humanized antibody directed towards the ErbB2 protein, has exhibited utility in the treatment of some individuals with breast cancer (Shak, 1999).

Two mechanisms for ErbB receptor activation in human tumors have been described. First, it is known that the overexpression of receptors results in their constitutive activation, either by facilitating the formation of the active dimeric form of the receptors or by swamping phosphatases that keep basal tyrosine kinase activity in check. In fact, overexpression of one of the ErbB family members, ErbB2, correlates with a poor prognosis of individuals with breast cancer (Slamon et al., 1989). ErbB activation in tumors also arises from autocrine activation mechanisms where cells produce and secrete EGF-like growth factors (Normanno et al., 1994). For both mechanisms it is thought that receptor activation leads to receptor autophosphorylation on tyrosine

residues, which in turn triggers cascades of intracellular events culminating in tumor cell growth. An effective ErbB-directed anti-tumor agent would suppress ErbB signalling arising from both overexpression and autocrine stimulation mechanisms.

Like its vertebrate homologs, the *Drosophila* EGF Receptor (DER) mediates various inductive signalling events in many developmental processes to regulate proper cell specification and tissue patterning (Ray and Schüpbach, 1996; Perrimon and Perkins, 1997; Schweitzer and Shilo, 1997). During developmental processes, DER signalling activity is precisely controlled by the carefully orchestrated deployment of the activating and inhibiting ligands (for reviews, see Perrimon and McMahon, 1999; Freeman, 2000). So far, four activating ligands have been identified: Vein (Vn) (Schnepp et al., 1996), Spitz (Spi) (Rutledge et al., 1992), Gurken (Grk) (Neuman-Silberberg and Schüpbach, 1993) and Keren (Reich and Shilo, 2002; Urban et al., 2002), each of which possesses an EGF repeat similar to that of transforming growth factor α (TGFα), a known ligand of the vertebrate EGFR. In addition, DER signalling can be regulated by negative factors such as Argos (Aos) (Schweitzer et al., 1995), Sprouty (Spry) (Casci et al., 1999) and Kekkon 1 (Kek1) (Ghiglione et al., 1999).

kek1 was isolated in a screen for genes whose expression overlaps that of activated DER during oogenesis (Musacchio and Perrimon, 1996; Ghiglione et al., 1999). It was shown to be transcriptionally regulated by DER and the Ras/MAPK pathway in follicle cells (Ghiglione et al., 1999). In developmental assays, the loss of *kek1* activity was associated with an increase in DER activity, whereas ectopic overexpression of the gene suppressed receptor activation, strongly suggesting that the Kek1 protein acts as a feedback negative regulator of DER activity. Consistent with this model, epistasis studies placed the function of Kek1 upstream of DER (Ghiglione et al., 1999). This, together with the observation that Kek1 encodes a single-pass transmembrane protein containing six contiguous Leucine-Rich Repeats (LRR) and one Immunoglobulin (Ig)-like domain (Musacchio and Perrimon, 1996), suggests that Kek1 acts at the cell surface to suppress DER signalling. Indeed, our previous studies indicate that Kek1 is capable of physically interacting with DER.

Despite the crucial role of Kek1 in controlling the level of DER activity during oogenesis, little is known about the precise mechanism by which Kek1 antagonizes this RTK. The purpose of the present study was to better understand the Kek1 inhibitory mechanism and to ascertain whether this regulation is tissue specific. In addition, we wanted to determine whether Kek1 expression could antagonize the growth of human and mouse mammary tumor cells through the suppression of ErbB receptors signalling. We show that as is the case during oogenesis, Kek1 antagonizes DER activity in the wing and eye imaginal discs through a negative feedback loop. Furthermore, we show that Kek1 and DER/ErbB form heterodimers, preventing activating ligands to bind to these receptors, and that this interferes with autophosphorylation and signal transduction of the receptors. Finally, we show that Kek1 may be employed as an inhibitor of mammalian ErbB receptors.

MATERIALS AND METHODS

Fly stocks

The following *Drosophila* lines were used: CY2-Gal4 (Queenan et al., 1997), MS1096-Gal4 (Capdevila and Guerrero, 1994), GMR-Gal4 (Freeman, 1996), nos-Gal4 (Rorth, 1998), T155-Gal4 (Harrison et al., 1995), en-Gal4 (Tabata et al., 1995), UAST-*DERDN* (Freeman, 1996), UAST-*λtop* (Queenan et al., 1997), UAST-*kek1* and UAST-*kek1TM* (Ghiglione et al., 1999), $egfr^{QYI}$ (Clifford and Schüpbach, 1989), grk^{2B} (Neuman-Silberberg and Schüpbach, 1993), $Ellipse^{1}$ (Baker and Rubin, 1989). The *kek1* lines *15A6 (kek1-lacZ)*, *RA5* and *RM2* are described elsewhere (Musacchio and Perrimon, 1996; Ghiglione et al., 1999).

Construction of *kek1* plasmids and generation of transgenic lines

Cloning details for making these deletion constructs are available under request. After mutagenesis by PCR, the coding regions were subcloned into the P-element vector pUAST (Brand and Perrimon, 1993) or pUASP (Rorth, 1998). All these *kek1* constructs contain two consecutive Myc epitope tags in frame to the C terminus. ΔNT, ΔNT+LRR, ΔNT+LRR+Ig, ΔLRR, ΔIg are deletions in the *kek1* extracellular domain from amino acids 23 to 88, 23 to 277, 23 to 430, 126 to 277, 329 to 430, respectively. *kek1sec* was made by introducing a stop codon immediately before the TM domain (at amino acid 446).

The *kek1/DER* chimeric constructs were made by fusing the extracellular and TM domains of different *kek1* deletions described above (amino acids 1 to 473) to the *DER* intracellular domain (at amino acids 744, immediately after the TM domain).

The UAS constructs were introduced into w^{1118} flies by standard methods of P-element-mediated germline transformation (Spradling, 1986). For each construct, several independent transgenic lines were generated and tested.

Antibody staining

Embryos and third instar wing imaginal discs were collected, fixed and stained (Bilder and Perrimon, 2000), using the monoclonal mouse anti-Myc antibody (Ab-1, Calbiochem). Tissues were co-stained with anti-FasIII to reveal embryonic basolateral membranes, and rhodamine-phalloidin to reveal the apical surface of imaginal discs. Confocal images were collected on a Leica TCS confocal microscope.

For detection of the β-galactosidase activity, imaginal discs were dissected in PBS and fixed in 1% glutaraldehyde for 20 minutes. Imaginal discs were then stained with 1 mg/ml X-Gal in X-Gal staining buffer at 37°C for 2 hours.

Sf9 cells on cover slips were co-infected at an M.O.I. of 0.2 with baculovirus encoding EGFR and Kek1. Cells were then fixed in methanol, incubated with rabbit anti-EGFR (1005, Santa Cruz) and mouse anti-Myc Ab-2 antibodies, and stained with FITC-goat-anti-mouse IgG and rhodamine-goat-anti-rabbit IgG (Jackson Immunochemicals).

Sf9 insect cells experiments

Recombinant baculoviruses encoding DER and Kek1 have been described previously (Ghiglione et al., 1999). The Kek1 versions ΔLRR and ΔIg described above were subcloned into the baculovirus transfer vector pVL1392 (Pharmingen) and recombinant baculoviruses were produced as described previously (Ghiglione et al., 1999).

For co-immunoprecipitation studies between DER and Kek1ΔLRR or Kek1ΔIg, we proceeded as described previously (Ghiglione et al., 1999). Immunoprecipitations from lysates were carried out using anti-Myc Ab-2 or anti-DER (a generous gift from M. Freeman) antibodies. Precipitates were blotted with anti-DER. Filters were then stripped and reprobed with anti-Myc.

For [^{125}I]EGF crosslinking experiments, insect cells were infected as described above and incubated for 5 minutes at room temperature with 0.5 μCi [^{125}I]EGF (Amersham) in the absence and presence of excess cold EGF (30 nM). Bis-sulfosuccinimidyl suberate (BS^3) was added to 1 mM and the incubation was continued for another 30 minutes. Cells were then lysed and immunoprecipitated as above, and

the radioactive bands were visualized using a Molecular Dynamics Storm Phosphorimaging system.

Mammalian cell lines

Mouse mammary tumor cell lines IJ9921 (MMTV-heregulin/NDF), NF-639 (MMTV-neu) and AC-816 (MMTV-v-Ha-ras) were derived from mammary tumors from transgenic mice and have been described previously (Krane and Leder, 1996; Muller et al., 1988; Sinn et al., 1987). MDA-MB-468 human mammary tumor cells were from ATCC, and HEK293-Ecr human embryonic kidney cells expressing the ecdysone receptor for inducible protein expression were obtained from Invitrogen. Cells were routinely grown in DMEM supplemented with 10% bovine calf serum (Gibco-BRL), 4 mM glutamine (Bio-Whittaker), penicillin (50 U/ml) and streptomycin (50 mg/ml) (Sigma).

Plasmids and transfections

The *kek1* cDNA was subcloned into pcDNA3.1 and pIND expression vectors (Invitrogen) adding either a Myc or a HA epitope tag in frame to the C terminus. The Kozak sequence was also changed from the native *Drosophila* sequence to the consensus mammalian sequence CCACCAUGG to achieve optimal expression in mammalian cells (Kozak, 1987). Stable transfectants were generated by lipofection (Lipofectamine Plus^{TM}, Gibco-BRL) and selection in G418 for 2-4 weeks. Kek1 expression in picked clones was verified by western blotting and/or immunoprecipitation using anti-Myc (Ab2, NeoMarkers) or anti-HA epitope (Ab Y11, Santa Cruz Biotechnology) antibodies.

Cell growth and transformation assays

Growth assays were performed by seeding 500-2000 cells in 24-well plates with DMEM media/10% FBS. Time points were taken at days 2, 4, 6 and 8 by trypsinization and counting. Anchorage-independent growth assays were performed by suspending 10^4 cells in 0.36% Bactoagar (Difco) over a 0.6% agar base layer in DMEM/10% FCS in 35 mm dishes. Every four days, ~300 μl of media was added to each plate. After 2-3 weeks, colonies were stained overnight with 0.5 mg/ml nitrobluetetrazolium (NBT, Sigma) in PBS and counted. Each experiment was performed in triplicate and repeated at least two times. In vivo transformation was measured by injecting 10^6 cells subcutaneously behind each front leg of nude mice. Tumors were excised 3-4 weeks later and weighed. Each experiment was performed at least three times.

293 cell co-immunoprecipitation and immunoblotting experiments

HEK293-Ecr cells expressing HA-tagged Kek1 were treated without or with 5 μM Ponasterone A (Invitrogen) for 20-24 hours to induce Kek1 expression. For EGF stimulation, experiments cells were serum-starved for another 4 hours and then treated without or with 50 ng/ml EGF (Sigma) for 5 minutes at 37°C. Equivalent protein amounts of cleared lysates were immunoprecipitated with 1.5 μg anti-phosphotyrosine (4G10, Upstate Biotechnology), anti-EGFR (Ab-1, NeoMarkers) or anti-HA epitope. Precipitates were resolved by SDS-PAGE, transferred to nitrocellulose and blotted with anti-HA or anti-EGFR (1005, Santa Cruz) antibodies. Blotted proteins were detected using horseradish peroxidase-coupled secondary antibody followed by enhanced chemiluminescence. Erk1/2 activation was measured by blotting lysates with an anti-phospho-Erk1/2 (Thr202/Tyr204) antibody according to the instructions of the manufacturer (New England Biolabs), and levels were correlated with total Erk2 protein detected with an anti-Erk2 antibody (sc-1647, Santa Cruz).

RESULTS

kek1 negatively regulates DER activity and acts in a feedback loop in the wing and eye imaginal discs

Previously, we showed that *kek1* negatively regulates DER activity and acts in a feedback loop during oogenesis (Ghiglione et al., 1999). To extend these observations to other tissues, we examined the expression of *kek1* in the wing and eye imaginal discs by using the 15A6 enhancer trap line (*kek1-lacZ*) that faithfully mimics the *kek1* RNA expression pattern in the embryo and the follicle cells (Musacchio and Perrimon, 1996; Ghiglione et al., 1999). In the wing disc, *kek1-lacZ* is expressed in vein primordia and in the L3/L4 intervein region (Fig. 1A). In the eye disc, *kek1-lacZ* expression is detected behind the morphogenetic furrow (Fig. 1D). These expression patterns correlate with dpERK activation in these tissues and some of the known requirements for DER activity (Gabay et al., 1997a; Gabay et al., 1997b), thus strongly suggesting that *kek1* expression is dependent on DER signalling in imaginal discs. To test this hypothesis, we examined the expression of *kek1-lacZ* in either loss- or gain-of-function DER signalling. The expression of *kek1-lacZ* was strongly reduced following expression of a dominant-negative form of the receptor (Fig. 1C,F), and an increased number of cells expressing *kek1* were detected in the presence of an activated form of the receptor (Fig. 1B,E). Thus, as observed during oogenesis, *kek1* expression is dependent on DER activation in the eye and wing imaginal discs.

Flies carrying a deletion of the *kek1* gene are viable and fertile (Musacchio and Perrimon, 1996), although the eggs and embryos derived from *kek1* mutant females are weakly dorsalized as a result of the weak hyperactivation of DER signalling (Ghiglione et al., 1999). To gain insights into the possible function of *kek1* during imaginal discs development, we

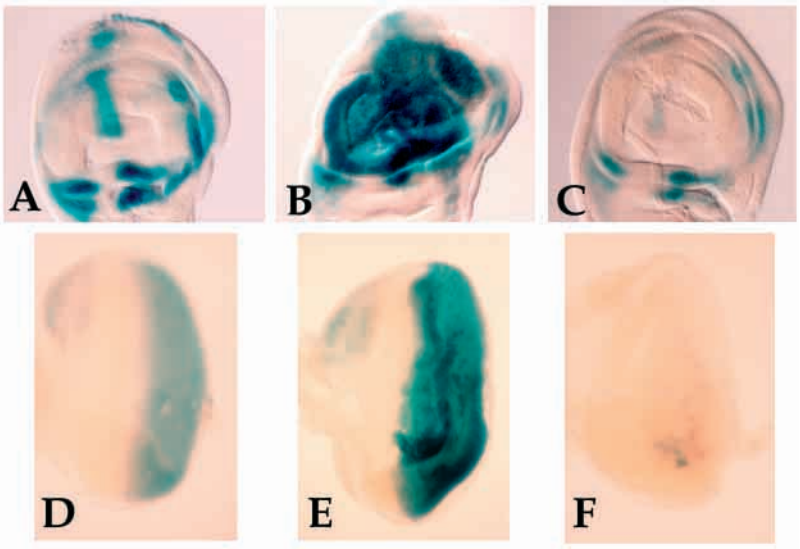

Fig. 1. *kek1* expression in wing and eye discs. Expression of the *kek1-lacZ* enhancer trap line in the third instar wing and eye discs (A and D, respectively). Ectopic expression of UAS-*DERDN* in the wing pouch using MS1096-Gal4 and behind the morphogenetic furrow of the eye disc using GMR-Gal4 strongly reduce *kek1-lacZ* expression (C and F, respectively). Conversely, expression of an activated DER (UAS-*λtop)* using the same drivers leads to an expansion of *kek1-lacZ* expression in the corresponding domains of the wing (B) and eye discs (E).

carefully analyzed the phenotype of the adult wing and eye of *kek1* mutant flies and found that these structures do not have any overt morphological defects (Fig. 2B,J). To further probe the functional relationship between Kek1 and DER in discs, we examined the effects of removing *kek1* activity in flies heterozygous for the *Ellipse* (*Elp*) gain-of-function allele of DER (Baker and Rubin, 1989). *Elp* weakly increases DER signalling and suppresses ommatidia development in the eye and induces moderate ectopic vein development in the wing (Fig. 2C,E,K). Interestingly, reducing by half or completely removing *kek1* activity strongly enhances the *Elp* phenotypes (Fig. 2D,L). These dominant enhancer activities of *kek1* are similar to the effect of *Gap1* or *echinoid* mutations, two known negative regulators of DER signalling pathway (Bai et al., 2001).

Consistent with the conclusion that Kek1 is an inhibitor of the DER pathway in the imaginal discs, ectopic overexpression of UAS-*kek1* in the wing pouch using the MS1096-Gal4 driver causes a vein loss phenotype (Fig. 2M), as observed when UAS-*DERDN* is expressed using the same driver (Fig. 2N). Similarly, UAS-*kek1* or UAS-*DERDN* overexpression in the eye using the GMR-Gal4 line substantially reduces the number of photoreceptors and the eye size (Fig. 2F-H).

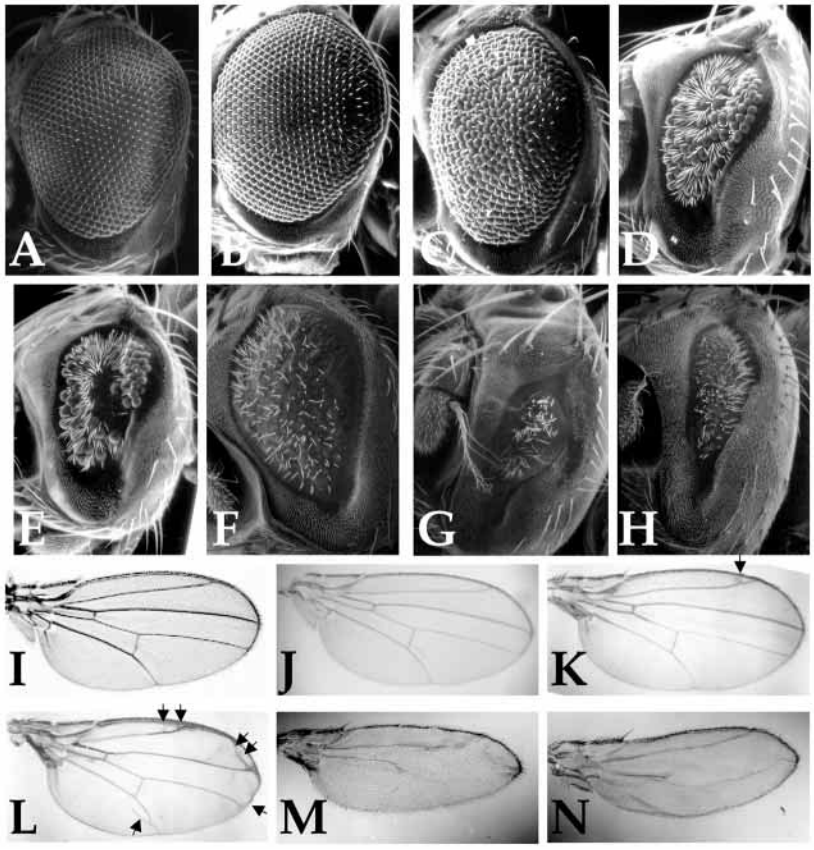

Fig. 2. Kek1 antagonizes DER activity in the eye and wing. (A-H) Eye phenotypes. (A) A wild-type adult eye posses around 750 ommatidia arranged in a highly ordered pattern. (B) Eyes from *kek1* mutants look wild type. (C) *Elp*/+ eyes are rough and this phenotype is strongly enhanced when homozygous for *kek1* (D). *Elp/Elp* eye (E). UAS-*kek1*/GMR-Gal4 (F) and UAS-*(kek1)*^{2}/GMR-Gal4 (G) are rough and reduced in size, similar to UAS-*DERDN*/GMR-Gal4 eyes (H). (I-N) Wing phenotypes. (I) A wild-type adult wing with its five longitudinal veins and two crossveins. (J) Wings from *kek1* mutants look wild type. (K) *Elp*/+ wings have a weak extra wing vein phenotype (arrows), and this phenotype is enhanced when homozygous for *kek1* (L). Overexpression of UAS-*kek1* using MS1096-Gal4 results in severe reduction in the vein material (M), similar to UAS-*DERDN* overexpression (N).

The LRR domains of Kek1 are critical for its function

Both Ig-like and LRR domains are present in the extracellular domain of Kek1. To determine the contribution of these domains in Kek1 activity, we generated a series of deletion constructs that removed some of these regions (Fig. 3A). These Kek1 truncated proteins retained the signal peptide, and their ability to inhibit DER signalling was assessed in overexpression assays in the follicle cells and in the wing discs using CY2-Gal4 or MS1096-Gal4, respectively.

The deletion of the N-terminal region or the Ig-like domain did not affect Kek1 function in these assays. Indeed, the eggs laid by females UAS-Δ*NT*/CY2-Gal4 or UAS-Δ*Ig*/CY2-Gal4 were strongly ventralized, and the wings of the UAS-Δ*NT*/MS1096-Gal4 or UAS-Δ*Ig*/MS1096-Gal4 adults have a strong vein loss phenotype, as observed after UAS-*kek1* overexpression in these tissues (Fig. 3A; data not shown). However, the eggs and adult wings obtained after overexpression of any of the proteins containing LRR domains deletion (ΔLRR, ΔNT+LRR, ΔNT+LRR+Ig) are wild type (Fig. 3A; data not shown), indicating that the deletion of these LRR domains is sufficient to completely abolish Kek1 function. Similarly, the deletion of the cytoplasmic domain (Kek1TM) or the cytoplasmic and transmembrane domains (Kek1sec), strongly or completely affect the Kek1 inhibitory ability, respectively (Fig. 3A; data not shown).

We previously showed in Sf9 cells that Kek1TM, but not the Kek1 intracellular domain, is able to bind to DER as efficiently as Kek1 (Ghiglione et al., 1999). To define this interaction more precisely, we tested which different truncated Kek1 proteins were able to bind to DER. After co-expression of a Myc-tagged version of these truncated proteins and DER in Sf9 cells, Kek1 was immunoprecipitated from the cell lysates using an anti-Myc antibody. Co-precipitations between Kek1ΔIg or Kek1 and DER were observed by probing the resulting blot with the anti-DER antibody (Fig. 3B, lane 3 and 4, respectively). However, Kek1ΔLRR was not able to co-precipitate DER (Fig. 3B, lane 2), suggesting a selectivity of the Kek1 LRR domains for binding to DER. Altogether, our structure-function analysis demonstrates the importance of the LRR domains for Kek1 function. These domains allow Kek1 to bind to DER, and this physical interaction is necessary for the inhibition of the receptor.

Kek1 and DER form heterodimers in vivo

To determine whether the physical association between Kek1 and DER occurs in vivo, we generated a series of Kek1-DER chimeras in which the whole extracellular and transmembrane part of the receptor is replaced

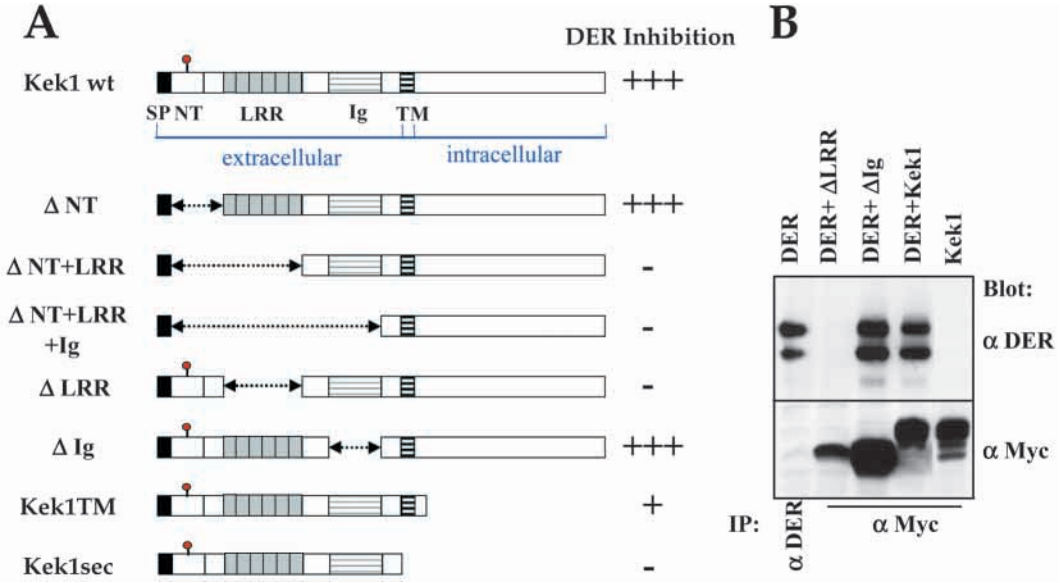

Fig. 3. Structure-function analysis of Kek1. (A) Schematic representation of the different Kek1 constructs: SP, signal peptide; NT, N-terminal region; LRR, Leucine-Rich Repeat domains; Ig, Immunoglobulin-like domain; TM, transmembrane domain. The relative efficiencies of these truncated proteins to inhibit DER signalling after overexpression in the follicle cells and the wing imaginal discs are indicated. (B) In vitro association between Kek1 and DER. Sf9 cells were infected with baculovirus encoding DER and co-infected with nothing (lane 1) or viruses encoding Myc-tagged versions of wild-type Kek1 (lane 4), Kek1ΔLRR (lane 2), Kek1ΔIg (lane 3). Lane 5 is a control with Sf9 infected with baculovirus encoding Kek1-Myc alone. Anti-DER and anti-Myc immunoprecipitates, lane 1 and lane 2-5, respectively, were blotted with anti-DER (upper panel) and then reprobed with anti-Myc (lower panel).

with the corresponding regions of Kek1 (Fig. 4A). Indeed, if Kek1 interacts with DER, then the chimera should promote heterodimerization with the endogenous receptor, hence its signalling activation. Overexpression of the Kek1-DER chimera in follicle cells was associated with hyperactivation of the DER pathway because the derived eggs were strongly dorsalized (Fig. 4C). A similar phenotype was obtained after overexpressing the Kek1ΔIg-DER chimera (Fig. 4E) but not Kek1ΔLRR-DER (Fig. 4D), thus confirming the importance of the LRR domains for Kek1 function.

We then tested whether the Kek1-DER chimera leads to DER hyperactivation through interaction with the endogenous receptor or through homodimerization of the chimeric protein. To distinguish between the two models, we overexpressed this chimera in *DER*^{top} mutant females. The eggs obtained after overexpression of the Kek1-DER chimera in *top* homozygous mutant females were as strongly ventralized as those laid by *top* females (Fig. 4F,G) (Schüpbach, 1987), indicating that endogenous DER is required for Kek1-DER chimera activity. Consistent with the hypothesis that Kek1-DER forms a heterodimer with DER and not a homodimer, we obtained ventralized eggs after overexpressing a Kek1-Btl chimera in the follicle cells (data not shown), Btl being another *Drosophila* RTK (Glazer and Shilo, 1991).

Altogether, we conclude that Kek1 is able to form heterodimers with DER in vivo, and that this association inhibits DER activity.

Kek1 subcellular localization

Deletion of its cytoplasmic domain strikingly decreases the ability of Kek1 to inhibit DER (Fig. 3A) (Ghiglione et al., 1999). As the Kek1 cytoplasmic domain is not implicated in the association with DER, we reasoned that it could possibly play a role in Kek1 subcellular localization.

To test this hypothesis, UAS-*kek1-myc* and UAS-*kek1TM-myc* were expressed in embryonic and imaginal wing disc epithelia using the en-Gal4 driver. First, we observed a complete and a weak inhibition of DER signalling by Kek1-Myc and Kek1TM-Myc, respectively (Fig. 5E,F), a result that is consistent with our previous results using untagged proteins. This indicates that the Myc epitopes do not interfere with Kek1 function. Interestingly, the subcellular localization of these proteins, visualized using an anti-Myc antibody, was strikingly different. Although expression of Kek1-Myc is clearly apical (Fig. 5A,C), expression of Kek1TM-Myc is basolateral (Fig. 5B,D), indicating that the intracellular domain of Kek1 is required for its correct subcellular localization. As DER has been shown to be apically located (Sapir et al., 1998), the aberrant localization of Kek1TM provides a likely explanation for its inability to inhibit the receptor efficiently when compared with the wild-type Kek1 protein.

Kek1 binds all known mammalian receptors of the ErbB/EGFR family

As Kek1 acts as a feedback inhibitor of DER signalling during *Drosophila* development, through a direct association between these two transmembrane proteins, we examined its ability to interact physically with mammalian ErbB family members. We first examined the interaction between Kek1-HA and human EGFR in transfected HEK293 cells, where Kek1-HA is robustly expressed in an all-or-nothing manner with addition of Ponasterone A (Pon A). Fig. 6A shows that Kek1-HA was co-immunoprecipitated with the endogenous EGFR only after expression was turned on with PonA. Likewise, EGFR was co-

immunoprecipitated with Kek1-HA after PonA treatment (data not shown). Similar results have been obtained using baculovirus/Sf9 insect cells (see Fig. 7A) and COS monkey cell expression systems (data not shown) using Myc-tagged Kek1, indicating that Kek1 has the ability to interact with mammalian EGFR. In addition, Kek1 is capable of interacting with the other three members of the ErbB receptor family (i.e ErbB2, ErbB3 and ErbB4; data not shown). Thus, the interaction between Kek1 and the mammalian receptors allows us to conduct a more detailed characterization of the biochemical mechanism underlying this interaction using reagents that are unique to the mammalian system.

Kek1 inhibits EGF mediated activation of the EGFR and downstream signalling pathways

To assess the effect of Kek1 association with EGFR/ErbB family members, we examined two of the early biochemical events associated with EGFR activation, receptor autophosphorylation and the stimulation of the Erk1 and Erk2 mitogen-activated kinases (MAPKs). In these experiments we treated HEK293-Ecr cells without or with PonA to induce Kek1-HA expression (Fig. 6B, lower panel) and then starved the cells for 4 hours without serum. We then treated the cells without or with EGF for 5 minutes. In the experiment shown in Fig. 6B (upper panel), we immunoprecipitated cell lysates with anti-phosphotyrosine antibodies to isolate tyrosine-phosphorylated proteins, and then blotted precipitates with anti-EGFR. In the absence of Kek1-HA expression, EGF potently stimulated the association of the EGFR with anti-phosphotyrosine. However, the expression of Kek1-HA resulted in a loss of the receptor from anti-phosphotyrosine precipitates, indicative of an inhibition of receptor autophosphorylation.

In Fig. 6C, we examined the stimulation of the Erk1 and Erk2 serine/threonine kinases by probing lysates from treated cells with an antibody that recognizes the phosphorylated (activated) forms of these proteins. We observed that Kek1-HA expression inhibited the activation of Erks in response to EGF by ~75% in the HEK293-Ecr cells. These observations indicate that, consistent with its activity in flies, Kek1 interacts with the mammalian EGFR to suppress receptor activation and signalling through the MAPK cascade.

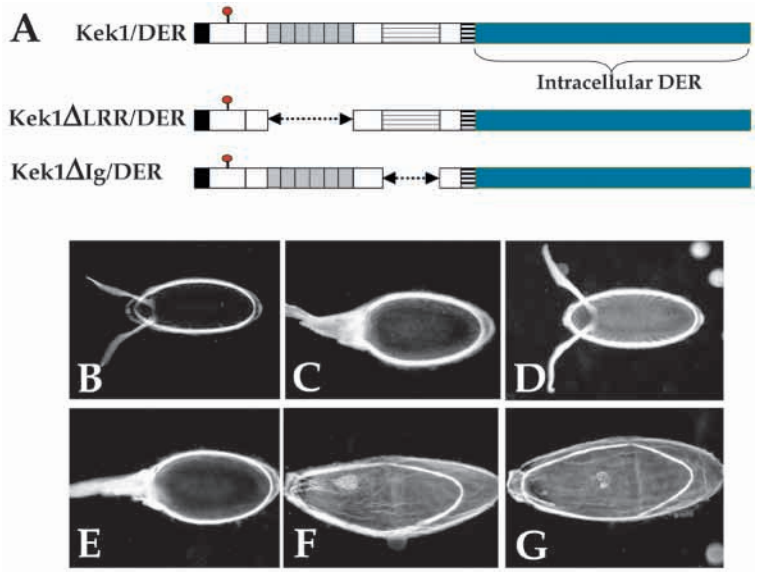

Fig. 4. Activity of Kek1-DER chimeras. (A) Schematic representation of the different Kek1-DER chimeras. (B) Wild-type egg with its two dorsal appendages. The eggs laid by females UAS-*kek1-DER*/T155-Gal4 (C) and UAS-*kek1ΔIg-DER*/T155-Gal4 (E) are strongly dorsalized. (D) Eggs laid by females UAS-*kek1ΔLRR-DER*/T155-Gal4 are wild type. Eggs obtained after overexpressing UAS-*kek1-DER* in the follicle cells of *top* homozygous females are strongly ventralized (F), similar to eggs laid by *top*/*top* females (G).

Kek1 inhibits ligand binding

To examine the mechanistic details underlying Kek1 suppression of EGFR activity, we used the baculovirus/Sf9 insect cell expression system. This system was employed because the viral infection allows tight control of both the relative levels of proteins expressed in each cell and the number of protein expressing cells.

In the experiment depicted in Fig. 7A, we tested the role of Kek1 on ligand binding and activation of the EGFR. Kek1-Myc or human EGFR were expressed alone, or the two proteins were co-expressed with EGFR in excess. Cells were treated without or with EGF, and lysates were immunoprecipitated with antibodies directed to either Myc epitope (lanes 1-4) or EGFR (lanes 5-9). When precipitates were blotted with anti-phosphotyrosine, we observed a strong stimulation of receptor autophosphorylation by the growth factor in anti-receptor precipitates (upper panel,

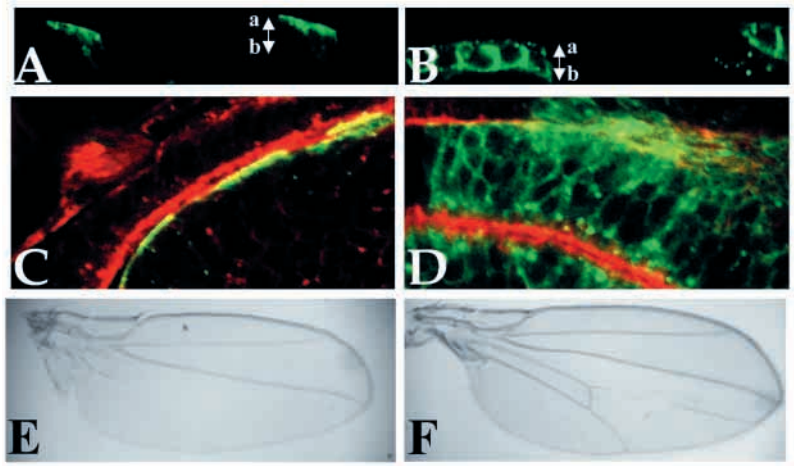

Fig. 5. The cytoplasmic domain of Kek1 is required for subcellular localization. (A-D) Confocal microscope sections showing anti-Myc immunostaining after overexpression of Myc-tagged UAS-*kek1* and UAS-*kek1TM* in embryos (A and B, respectively) and wing imaginal discs (C and D, respectively) by using en-Gal4 as a driver. Apicobasal polarity is shown (a↔b) with apical orientation upwards. Apical surface of wing imaginal discs is revealed with rhodamine-phalloidin. Anti-Myc staining is in green and rhodamine-phalloidin is in red. (E,F) Resulting UAS-*kek1*/en-Gal4 and UAS-*kek1TM*/en-Gal4 adult wings (compare with a wild-type wing in Fig. 2I).

lanes 6-9), indicating that the total EGFR population responded strongly to ligand treatment. However, although the presence of EGFR was apparent in the anti-Myc precipitates (middle panel, lanes 2 and 3), no stimulation of the tyrosine phosphorylation of this Kek1-associated population of receptors was observed (upper panel, lanes 2 and 3). Moreover, EGFR in anti-Myc precipitates were not capable of interacting with [^{125}I]EGF (lower panel, compare lane 2 with lanes 6 and 8).

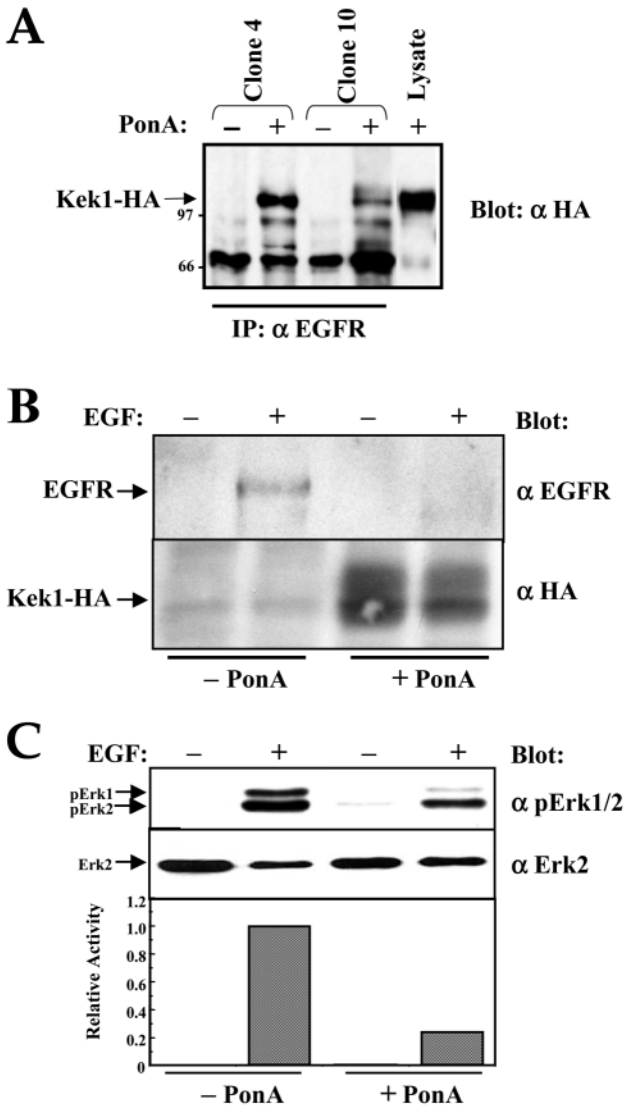

Fig. 6. Kek1 association and inhibition of human EGFR. (A) Association of Kek1-HA with human EGFR in two stably transfected HEK293-Ecr cell lines. Cells were treated without or with Ponasterone A (PonA) to induce Kek1-HA expression, and lysates were immunoprecipitated (IP) with antibodies to EGFR. Precipitates were then immunoblotted with anti-HA. Cell lysate (right lane) was included as a positive control for blotting. (B,C) Inhibition of human EGFR signalling in HEK293 cells by Kek1. 293-Ecr stably transfected cells (clone 4) were treated without and with PonA for 24 hours, and then treated without and with EGF as indicated. (B) Inhibition of EGFR autophosphorylation: upper panel, lysates from treated cells were immunoprecipitated with anti-phosphotyrosine antibodies; precipitates were blotted with anti-EGFR. Lower panel, lysates were blotted with anti-HA to detect Kek1-HA expression. (C) Inhibition of Erk1/2 activation: lysates from treated cells were blotted with antibodies specific for phosphorylated Erk1 and Erk2 (upper panel) and re-probed with antibodies that recognize the total Erk2 population (middle panel). Bands were quantified and relative Erk activity plotted (lower panel).

One possible explanation for the Kek1-mediated suppression of ligand binding and activation is that Kek1 becomes trapped in an intracellular compartment and retains a population of the EGFR. To examine this possibility we looked at the localization of Kek1-Myc and EGFR or DER by immunofluorescence in Sf9 cells and in egg chambers and found that the two proteins co-localize at the cell surface of the co-expressing cells (Fig. 7B; data not shown).

One additional line of evidence that Kek1 is acting at the cell surface comes from the fact that Kek1, after ectopic expression in the germline, is able to weakly inhibit DER that is expressed in the overlying follicle cells (Fig. 7C). This 'trans-inhibition' is greatly enhanced after removal one copy of *top* or one copy of the germline-specific ligand *grk* (Fig. 7D; data not shown).

Altogether, our results indicate that Kek1 directly interacts with EGFR/DER at the cell surface to inhibit ligand binding. These results are also consistent with other results that we have obtained in the fly assays showing that although Kek1 inhibits the ability of Grk and Spi to activate DER in various tissues, Kek1 does not physically associate with these ligands (data not shown).

Kek1 inhibits the growth of mammary cell lines with activated ErbB receptors

Our results demonstrate that the *Drosophila* Kek1 protein can act as a potent inhibitor of EGFR/ErbB in tissue culture cells. To extend this observation, we asked whether Kek1 could act as a suppressor of mammalian ErbB-mediated mammary tumor cell growth. We constructed a series of mammalian cell lines stably transfected with Kek1 and then compared the growth properties of Kek1 transfectants with control cells stably transfected with vector alone. Two human cell lines were examined: HEK293-Ecr transfectants, a human embryonic kidney cell line where Kek1 was expressed in an inducible manner, and MDA-MB-468 cells, a mammary epithelial cell line that overexpresses the EGFR. More importantly, the impact of Kek1 expression on a series of cell lines derived from oncogene-induced mouse mammary tumors from transgenic mice was also examined. IJ9921 cells were derived from expression of the EGF-like growth factor neuregulin 1 (Krane and Leder, 1996), NF-639 cells were from an activated form of ErbB2 (Muller et al., 1988), and AC-816 cells were derived from tumors induced by activated Ras (Sinn et al., 1987).

Kek1 transfectants exhibited a reproducibly slower growth rate than controls in four of the cell lines examined (Fig. 8A). Turning on Kek1 expression in HEK293 cells with the addition of PonA was sufficient to slow their growth, suggesting that differences in cellular growth rates in all lines is probably not a result of clonal variation. Moreover, these four Kek1 transfectants also exhibited a much lower tendency than controls to grow in soft agar and to grow as tumors when introduced into animals (Fig. 8B). The exception to this trend was the AC-816 cell line, which exhibited similar growth properties whether or not Kek1 was expressed.

These observations indicate that introduction of Kek1 into a subset of mammalian cell lines inhibits their cellular growth

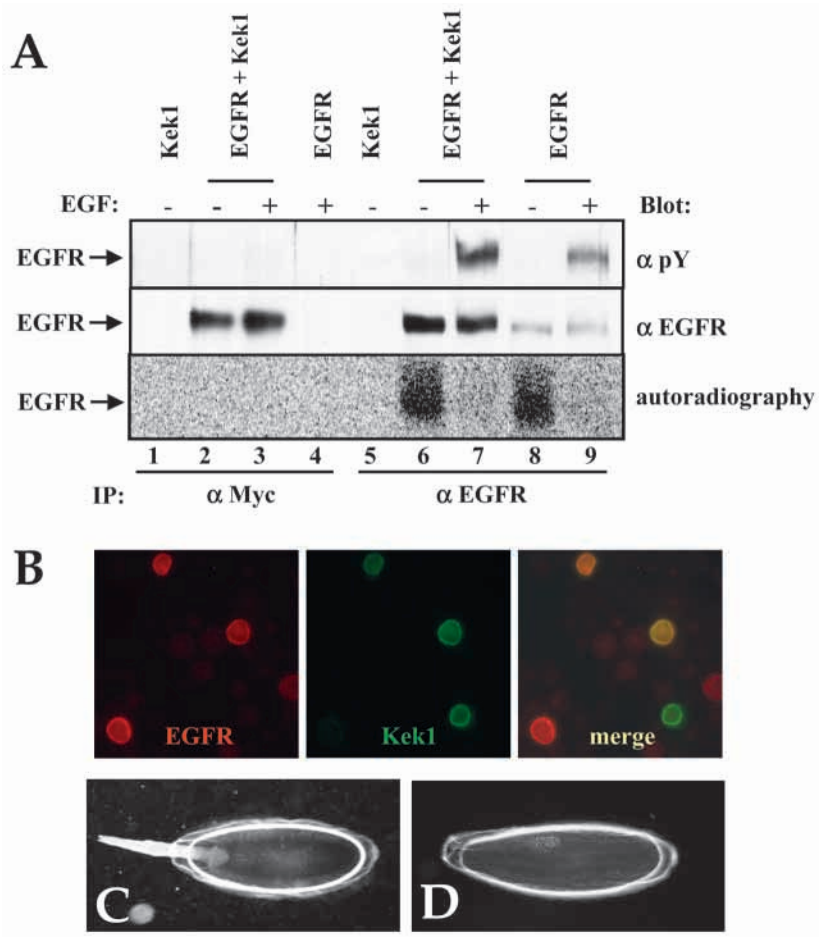

Fig. 7. Mechanism of EGFR inhibition by Kek1. (A) Inhibition of EGF binding and EGF-stimulated receptor tyrosine phosphorylation by Kek1. Sf9 insect cells were infected with baculoviruses encoding either Kek1-Myc or EGFR, or co-infected with both viruses. Cells were treated without or with 30 nM EGF as indicated. For the [^{125}I]-labeled EGF crosslinking experiment (lower panel), trace levels (0.1 nM) of iodinated growth factor and 1 mM BS^3 crosslinker were added to all samples at the time of EGF addition. Lysates from cells were immunoprecipitated with antibodies to either Myc epitope or to EGFR. Precipitates were exposed to autoradiography (lower panel), or were blotted with antibodies to phosphotyrosine (upper panel) or EGFR (middle panel). (B) Co-localization of Kek1 and EGFR at the cell surface. Sf9 insect cells were infected at a low multiplicity of infection with baculoviruses encoding Kek1-Myc and human EGFR. Cells were fixed and stained with both rabbit anti-EGFR (left panel) and mouse anti-Myc epitope (middle panel). Images were merged to show co-localization (right panel). (C,D) Kek1 can inhibit DER in trans. (C) 6% of the eggs (n=112) laid by females UASp-*kek1*/nos-Gal4 are weakly ventralized (partial or total fusion of the dorsal appendages). (D) Among the 64% of the ventralized eggs (n=96) laid by *top*/+; UASp-*kek1*/nos-Gal4 females, 8% are strongly ventralized.

properties. Consistent with a putative role in disrupting signalling at the cell surface, a cell line derived from tumors obtained through Ras activation were not susceptible to Kek1-mediated growth suppression.

DISCUSSION

In this study we demonstrate that the *Drosophila melanogaster* transmembrane protein Kek1 acts in a negative feedback loop in many DER-mediated developmental processes. Furthermore, we show that Kek1 is capable of binding to, and suppressing, the signalling functions of mammalian ErbB receptor family members. In particular, Kek1 suppresses ligand binding and autophosphorylation of these receptors, resulting in the suppression of downstream signalling events. An important functional outcome of these interactions is the suppression of tumor cell growth properties of mammalian cells. On the basis of these observations, we propose that there may exist mammalian Kek1 homolog(s) that act as negative modulators of ErbB receptors function. Thus, Kek1 or its homologs may represent good candidates as ErbB-directed anti-tumor agents, and may have some clinical utility.

Kek1 acts in a negative feedback loop to modulate DER activity in diverse tissues

kek1 was originally identified as a negative regulator of DER signalling in follicle cells (Ghiglione et al., 1999). We have extended these observations to two additional tissues, the eye and wing imaginal discs. We show that *kek1* is expressed in cells where DER activity is required and that *kek1* expression is lost in the absence of DER activity. Furthermore, we found that more cells express *kek1* following DER hyperactivation. Finally, we showed that, in a sensitized genetic background, Kek1 acts as a negative regulator of DER activity. These studies extend our previous findings and strengthen the functional relationship between Kek1 and DER.

Results from both biochemical experiments and in vivo tests revealed that the LRR domains of Kek1 are crucial for the association between DER and Kek1, and DER inhibition. Furthermore, the Kek1 cytoplasmic domain, which has previously been shown to play a role in the overall efficiency of DER inhibition, appears to be critical for the proper apical subcellular localization of Kek1 in epithelial cells. Interestingly, the Kek1 C terminus contains a concensus sequence for a PDZ domain-binding site that we have shown can bind the PDZ domains of proteins such as Disc-Large or Scribble (data not shown). Because these proteins are crucial for the organization of apicobasal cell polarity (Bilder et al., 2003), it is possible that Kek1 localization depends on these factors or related polarity cues. Interestingly, subcellular localization of Kek1 to the apical side may be coordinated with DER/ErbB subcellular localization as well, as PDZ-containing proteins have also been implicated in ErbB subcellular localization (reviewed by

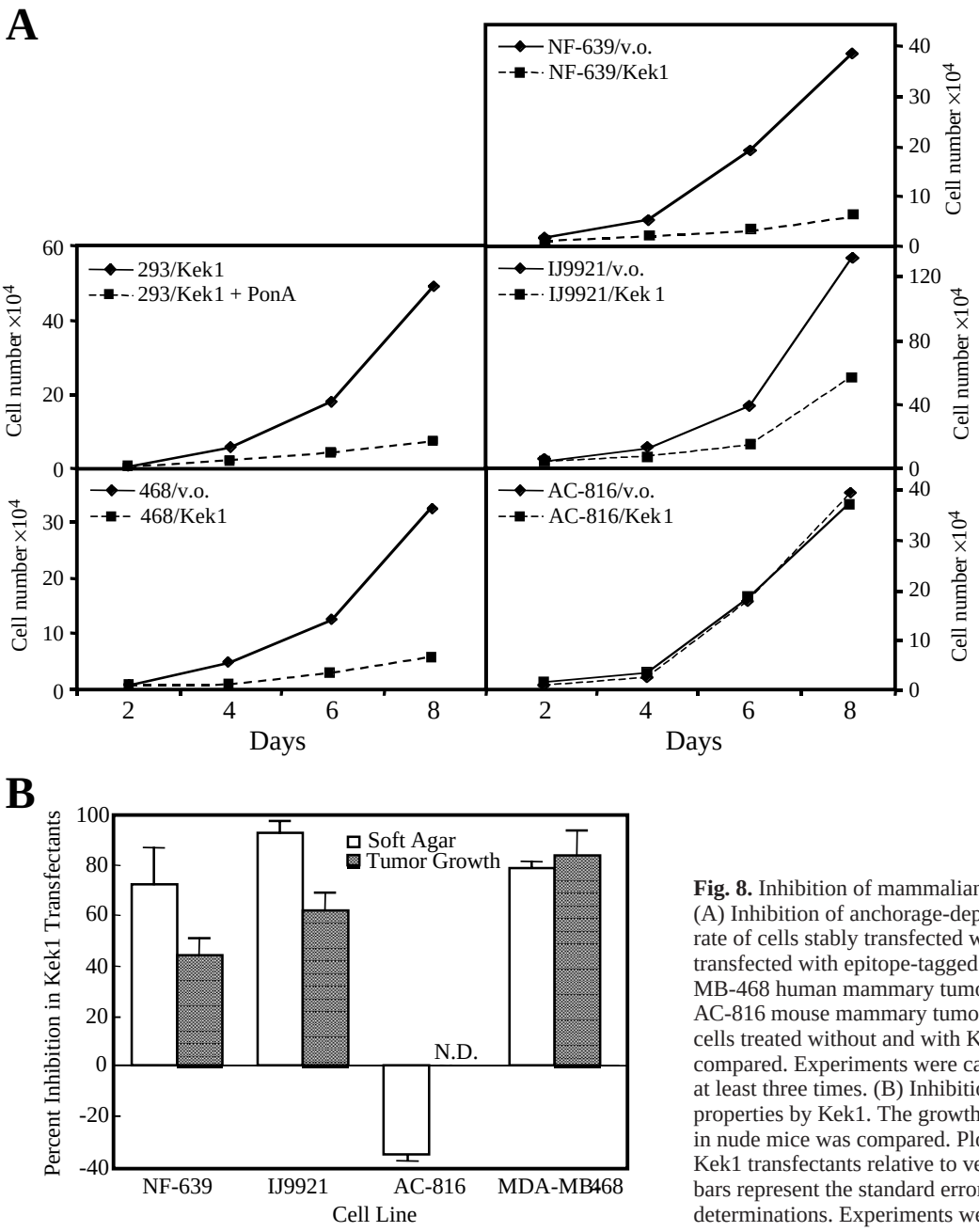

Fig. 8. Inhibition of mammalian cell growth by Kek1. (A) Inhibition of anchorage-dependent cell growth. The growth rate of cells stably transfected with vector alone (v.o.) or cells transfected with epitope-tagged Kek1 were compared for MDA-MB-468 human mammary tumor cells, and NF-639, IJ9921 and AC-816 mouse mammary tumor cells. Growth rates of HEK293 cells treated without and with Kek1 induction by PonA were also compared. Experiments were carried out in triplicate and repeated at least three times. (B) Inhibition of tumorigenic growth properties by Kek1. The growth of cells in soft agar or as tumors in nude mice was compared. Plotted is the percent inhibition by Kek1 transfectants relative to vector alone transfectants. Error bars represent the standard error of the mean of three to six determinations. Experiments were repeated at least three times.

Carraway and Sweeney, 2001). Further characterization of these interactions will be needed to clarify how subcellular localization of Kek1 and DER is regulated.

Mechanism of DER/ErbB binding and inhibition

Epistasis studies placed the action of Kek1 upstream of DER. As Kek1 is expressed in the same cell as DER, these observations suggest that Kek1 interacts with either the receptor to suppress its signalling function or with the ligand to sequester its activity. Our observations indicate that Kek1 can be co-immunoprecipitated with DER (Ghiglione et al., 1999) (this study) but not with its ligands (data not shown) suggesting that Kek1 interacts directly with receptors to interfere with ligand binding activity.

These findings are consistent with the biochemical interaction of Kek1 with all four mammalian ErbB receptor family members. When reconstituted in Sf9 insect cells, Kek1 blocked the binding of radiolabeled EGF to the population of EGFR associated with Kek1, but not the total receptor pool. Likewise, EGF-stimulated autophosphorylation of the Kek1-associated receptor population was blocked, but autophosphorylation of the total receptor pool was not. These observations suggest that Kek1 acts to suppress receptor signalling at least in part by physically interfering with ligand binding. However, other effects on receptor activation cannot be ruled out. We observed that Kek1 suppressed the growth properties of the NF-639 mouse mammary tumor cells, obtained from an activating point mutation in the transmembrane region of the ErbB2 receptor. As this mutation is thought to generate constitutive receptor tyrosine kinase

activity via a ligand-independent mechanism (Bargmann and Weinberg, 1988), it is likely that Kek1 also acts to interfere with receptor dimerization or other events necessary for its activity.

Our studies suggest that Kek1 is functionally similar to another *Drosophila* suppressor of DER signalling called Argos. Argos is also a transcriptional target of activated DER in developing tissues (Golembo et al., 1996; Wasserman and Freeman, 1998), and it has been demonstrated that Argos binds directly to DER to inhibit the binding of the natural ligand Spitz (Jin et al., 2000). However, the sequences of the two inhibitors are very distinct. Although Kek1 contains a series of LRR and Ig domains in its extracellular region, Argos contains an imperfect EGF-like domain (Freeman et al., 1992). Given that at least two proteins in the *Drosophila* genome are dedicated to a similar purpose, it seems likely that ErbB antagonists are also present in higher organisms.

Kek1-related genes?

Our previous studies indicated that the extracellular and TM region of Kek1 was sufficient to mediate its biological activity as well as its interaction with DER (Ghiglione et al., 1999). The present study indicate that the LRR domains of the extracellular region are necessary for the suppression of DER-mediated developmental events in flies. These results suggest that Kek1/receptor interactions are mediated by the LRR domains, pointing to LRR-containing extracellular proteins as candidates for mammalian Kek1 homologs. Numerous mammalian LRR proteins have been described and several have arrangements of subdomains similar to Kek1, including the Trk receptor tyrosine kinases (Shelton et al., 1995), LIG-1 (Suzuki et al., 1996) and a number of proteins of unknown function. The role of such proteins in ErbB-mediated developmental processes and tumor cell growth remains to be explored.

Particularly noteworthy is the small leucine-rich proteoglycan decorin, which has been shown to directly bind to human EGFR (Iozzo et al., 1999). However, although decorin is also a potent suppressor of tumor cell growth, its mechanism of action appears to differ from that of Kek1. Treatment of cells with soluble decorin induces the immediate tyrosine phosphorylation of the EGFR and subsequent signalling events (Moscatello et al., 1998; Patel et al., 1998), and sustained expression of decorin suppresses EGFR levels without affecting ligand binding activity. These results indicate that decorin is not functionally identical to Kek1. However, taken with our observations these data suggest that some LRR-containing extracellular proteins are capable of interacting with ErbB receptors to modulate their activities by multiple mechanisms.

Direct modulation of growth factor signalling

In a broader context, proteins such as Kek1 and decorin may be thought of as direct modulators of ErbB receptors that could assist in the integration of extracellular events with growth factor signalling. Numerous studies suggest that signalling through integrins, cell adhesion molecules and other cell surface proteins impact ErbB receptor signalling pathways, largely by influencing the extent to which various intracellular signalling pathways respond to receptor activation (Giancotti and Ruoslahti, 1999; Moghal and Sternberg, 1999). These examples represent indirect modulation of growth factor signalling through crosstalk between downstream components. We propose that LRR-containing proteins such as Kek1 and decorin are members of a larger functionally related class of glycoproteins that directly modulate growth factor signalling pathways by interacting with and influencing the properties of the receptors themselves (Carraway and Sweeney, 2001).

We thank members of the Leder laboratory, Harvard Medical School, for their helpful discussions and suggestions. We are also grateful to Thorkell Andresson, Robert Skraban, Holmfridur Thorsteinsdottir, Elin Gudmannsdottir and other members of the cancer genomics and proteomics groups at deCODE Genetics for their valuable comments and critical reading of the manuscript. We give special thanks to Kari Stefansson at deCODE Genetics for his goodwill and support of the work presented here. We thank Y. Paraiso and C. Arnold for generating the transgenic fly lines; N. Baker, M. Freeman, A. Michelson, P. Rorth, B. Shilo and T. Schüpbach for fly stocks and other reagents; and J. Duffy for sharing results before publication. This work was supported by NIH grant CA71702 (K.L.C.), Department of Defense Breast Cancer Research grants (K.L.C. and N.P.), la Ligue Nationale contre le Cancer, l'Association pour la Recherche sur le Cancer (ARC), le Ministère de la Recherche and l'INSERM (ACI-Biologie du Développement).

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