Development 130, 3725-3733

doi:10.1242/dev.00605

Echinoid is essential for regulation of Egfr signaling and R8 formation during Drosophila eye development

Susan A. Spencer and Ross L. Cagan

Department of Molecular Biology and Pharmacology, Washington University School of Medicine, 660 South Euclid Avenue, Campus Box 8103, St Louis, MO 63110, USA

Accepted 15 May 2003

SUMMARY

Precisely regulated Egfr activity is essential for normal development and cell differentiation. We demonstrate that the transmembrane protein Echinoid is required to downregulate Egfr activity in the developing *Drosophila* eye, ensuring a normal array of R8 photoreceptor neurons. Echinoid is an L1-type transmembrane molecule that is expressed in all cells of the eye imaginal discs and, unlike many other Egfr inhibitors, does not appear to be regulated transcriptionally. Echinoid co-precipitates with Egfr from cultured cells and eye imaginal discs, and Egfr activity promotes tyrosine phosphorylation of Echinoid. These observations suggest that Echinoid inhibits Egfr through direct interactions.

Key words: *Drosophila*, Echinoid, Egfr, R8, Retina

INTRODUCTION

Receptor tyrosine kinases play a central role in the development and patterning of epithelia. One of the best understood class of receptors is the epidermal growth factor receptor (Egfr) family, which has been implicated in control of mitosis (Zak and Shilo, 1992), morphogenesis (Duchek and Rorth, 2001) and differentiation (Freeman, 1996; Schweitzer and Shilo, 1997). Though broadly expressed, Egfr activation is tightly controlled through a number of positive and negative regulators. One important level of regulation is 'feedback inhibition', a common strategy in which receptor signaling triggers activation of inhibitory factors. In this paper, we present evidence that the transmembrane protein Echinoid plays such a role in the developing *Drosophila* retina.

Similar to most sensory systems, the developing fly eye employs a variety of patterning mechanisms to produce a precise arrangement of differentiated cells. It is a composite of some 750 unit eyes, or 'ommatidia', arranged into a perfect lattice, giving rise to the crystalline appearance of the adult compound eye. The precision with which this lattice forms makes the developing *Drosophila* retina an excellent system for identifying even modest alterations in important signaling pathways. Within the larval eye disc, the first cell to emerge within each ommatidium is the R8 photoreceptor. Differentiating R8s arise in rows within the morphogenetic furrow, each cell separated from its neighbor by 10-12 undifferentiated cells, and each row of cells precisely out of register with those in the row posterior to it (Fig. 1A-C). Once it emerges, each R8 cell begins recruitment of the other cell types necessary to form a mature ommatidium.

R8 differentiation relies on expression of the bHLH transcription factor Atonal. Initially expressed at low levels at the anterior edge of the morphogenetic furrow, Atonal is upregulated within the morphogenetic furrow in groups of six to ten cells known as proneural clusters. These groups are the first indication of R8 (and ommatidial) patterning in the developing eye disc (Jarman et al., 1994; Sun et al., 1998). Near the posterior edge of each cluster, the nuclei of two or three cells rise apically to form the R8 equivalence group (Dokucu et al., 1996). From these, a single cell will continue to express Atonal and differentiate as the R8 photoreceptor, eventually expressing the terminal R8 marker Boss.

The cells that comprise the proneural clusters exhibit high levels of phosphorylated Rolled/ERKA/MAP kinase, which constitutes a readout of Egfr activity. Rhomboid and Roughoid are activators of Egfr signaling that are expressed by cells at the posterior edge of the proneural clusters, and are responsible for the phosphorylated MAP kinase observed within these cells (Wasserman et al., 2000). This activation of the Egfr pathway appears to be short lived, coinciding precisely with the expression of Atonal. This period of activation is essential for R8 patterning: both hyperactivation and loss of Egfr function disrupt R8 differentiation and/or spacing (Baker and Rubin, 1989; Freeman, 1996; Dominguez et al., 1998; Spencer et al., 1998). Patches of developing eye tissue lacking Egfr activity show a loss of well-defined proneural clusters, a loss of proper R8 spacing and an overall decrease in the number of R8 cells as assessed by Boss expression (Dominguez et al., 1998). Conversely, transient overactivation of Ras pathway signaling increases Atonal expression throughout the morphogenetic furrow and prompts differentiation of additional R8 cells (Spencer et al., 1998). This latter result highlights the importance of limiting Egfr activity in the morphogenetic furrow to restrict the number of R8s.

The precise level and duration of Egfr activity is subject to

several levels of control. A number of inhibitors of Egfr have been described in *Drosophila*, such as Argos (Schweitzer et al., 1995), Kekkon (Ghiglione et al., 1999) and Sprouty (Casci et al., 1999). Each of these factors is transcribed in response to Egfr signaling, but none has been shown to play a role in limiting Egfr activity during R8 development. Egfr signaling is also attenuated by activity-dependent endocytosis, and proteins that control this process, such as *cbl*, are essential for maintaining Egfr signaling of appropriate duration (Levkowitz et al., 1998; Pai et al., 2000). In this paper, we present evidence that the transmembrane protein Echinoid (Bai et al., 2001) plays an essential role in R8 selection and retinal development by downregulating Egfr signaling after an initial period of activation. We further demonstrate that Echinoid and Egfr associate with one another and that Echinoid is a substrate for tyrosine phosphorylation in response to Egfr activity, suggesting a direct role for Echinoid in limiting Egfr signaling within the developing retina.

MATERIALS AND METHODS

Fly stocks

l(2)k01102 was obtained from the Berkeley Drosophila Genome Project. An additional lethal mutation was removed from the second chromosome by recombination with *al dp bw*; reversion analysis was performed on the resulting *l(2)k01102 dp bw* chromosome. The *echinoid dp* FRT line was created by recombination with *FRT40A* (Bloomington stock Center) and clonal patches of cells created by crossing *ed dp* FRT flies to *P{ry+7.2=hs-FLP}*; *ubiGFPFRT40A* flies (Bloomington Stock Center). Transgenic lines containing *UAS-edFLAG* and *UAS-edΔCFLAG* were created by standard procedures; UAS constructs were expressed under the actin promoter in clonal patches of cells by using the FLP-out cassette *P[w^{+mc}=AyGAL4]25 P[w^{+m}=UAS-GFP.S65T]T2/CyO* and *P{ry+7.2=hs-FLP}*, both from the Bloomington Stock Center. *Ellipse/CyO* flies were a gift from Nick Baker; other alleles of *echinoid*, *ed[slH8]/CyO* and *ed[slA12]/CyO* were gifts from J.-C. Hsu. *hs-rhomboid* was a gift from M. Ben-Shilo; *hs-argos* was a gift from H. Okano.

Constructs

echinoid (EST clone GM09285) was tagged with a FLAG epitope (*edFLAG*) or Myc epitope (*edmyc*) at the C terminus and cloned into the UAST vector for creation of transgenic flies, or into the heat-shock CaSpeR vector for expression in cultured S2 cells. EdFLAGΔC, which lacks the tyrosine-rich region of the Echinoid intracellular domain, was created by deleting amino acids 1078-1332 and fusing a FLAG epitope to the C terminus. EdFLAGΔN, which lacks the Ig and FN3 domains (but not the signal sequence), was created by deleting amino acids 69-787 and tagging the C terminus with a FLAG epitope. Egfr in hs-CaSpeR (hs-Egfr) and secreted Spitz in hs-CaSpeR (hs-sspi) were gifts from B. Shilo. Morgue-FLAG in pRMHa3 was a gift from Rebecca Hays. ERK-myc in pPAC was a gift from Tina Tootle. Ras^{Val12} in pIE1-3 was a gift from Callie Craig.

Antibodies and immunohistochemistry

Antibodies directed against Atonal (used 1:5000, gift from Y. Jan), Boss (used 1:2000, gift of Larry Zipursky), Senseless (used 1:1000, gift of H. Bellen) and dually phosphorylated ERKA (used 1:500, ERK; Sigma) were used to stain eye imaginal discs as described previously (Spencer et al., 1998): eye imaginal discs from third instar larvae were dissected into PBS and transferred immediately to 4% paraformaldehyde in PBS for 20 minutes. After washing twice in PBS and twice in PBS+0.3% TritonX-100 (Sigma), discs were incubated 4 hours at 4 degrees in primary antibody, PBS + 0.3% Triton X-100, 0.5 mg/ml BSA. After washing three times in PBS+0.3% TritonX-100, discs were further incubated in fluorescently tagged secondary antibodies [Cy3- or FITC-tagged anti-mouse and anti-rabbit, Jackson Immunochemicals; Alexa568-anti guinea pig (Santa Cruz) in PBS + 0.3% Triton X-100, 0.5 mg/ml BSA]. Antibodies against Corkscrew (CT-1 and CT-2; gift of M. Simon), phosphotyrosine (PY99-HRP; Santa-Cruz Biotechnology), Flag (HRP-linked M2; Sigma), Egfr (a gift from Nick Baker), C-terminal Echinoid (a gift from E. Rawlins and A. Jarman), N-terminal Echinoid (a gift from J.-C. Hsu) and Myc (rabbit polyclonal A-15; Santa-Cruz) were used for immunoprecipitations and western blot analysis.

Tissue culture and immunoprecipitations

S2 and SL2 cells were transiently transfected using Cellfectin (Cell Signaling). Transfection efficiency was monitored using pIE-1-Bgal. The amount of total heat-shock promoter in each transfection condition was kept constant by adding empty hs-CaSpeR vector to some reactions. Twenty-four hours after transfection, cells were heat-shocked three times for 30 minutes with 30 minute rests to prompt expression of genes in the hs-CaSpeR vector. Cells were washed once with room temperature PBS and lysed on ice with NP-40 lysis buffer: 1% NP-40, 50 mM Tris, pH 7.4, 1 mg/ml BSA, 1 mM Na-VO4, 1× Complete Protease Inhibitor cocktail. Cells were lysed at 4°C with mixing for 20 minutes, then centrifuged at 13,000 ***g*** for 15 minutes to remove particulate matter. Lysates were pre-incubated with ProteinA/Protein G Sepharose (Gamma-Bind, Pharmacia) for 30 minutes to reduce non-specific binding. Immunoprecipitations were carried out on lysates for 2-12 hours at 4°C using Sepharose linked to FLAG-M2 monoclonal antibodies (Sigma) or myc-A15 monoclonal antibodies (Santa Cruz). Beads were washed four times with lysis buffer and once with lysis buffer lacking BSA before eluting bound proteins with SDS-loading buffer. Novex 4-12% polyacrylamide gels were used for SDS-PAGE. Western analysis was essentially as described previously (Spencer et al., 1998); antibodies were used at the following concentrations: against Corkscrew (CT-1 and CT-2, 1:2000), phosphotyrosine (PY99-HRP; 1:500), Flag (HRP-linked M2; 1:500), Egfr (rabbit; 1:2000), N-terminal Echinoid (1:1000) and Myc (rabbit polyclonal A-15; 1:1000).

Immunoprecipitations from eye imaginal discs

Immunoprecipitations from tissue were carried out as described above with the following changes. Twenty third-instar larvae were obtained from each genotype: *w^{1118}*, *hs-rhomboid* (animals containing a transgene for heat-shock-induced *rhomboid* expression) or *hs-argos* (heat-shock-induced *argos* expression). Larvae were heat-shocked for 35 minutes in a 37°C water bath to induce transgene expression. Eye-antennal imaginal discs were immediately dissected from the animals in PBS and immediately transferred to NP-40 lysis buffer (described above). Discs were dissociated by drawing lysates up in a syringe with a 22-guage needle four times; lysates were otherwise treated as described for cultured cells.

RESULTS

echinoid is essential for R8 selection

Although tightly regulated Egfr activity appears necessary for normal R8 patterning and specification, many of the genes that provide the precise temporal and spatial regulation of receptor signaling are still unknown. To identify other genes involved in R8 selection, we screened P-element-mediated mutations for dominant modifiers of the *Egfr^{Ellipse}* phenotype. The P-element insertion *l(2)k01102* strongly enhanced the *Egfr^{Ellipse}* phenotype, resulting in an eye approximately one-third the size of *Egfr^{Ellipse}* heterozygotes (Bai et al., 2001) (Fig. 1D-F).

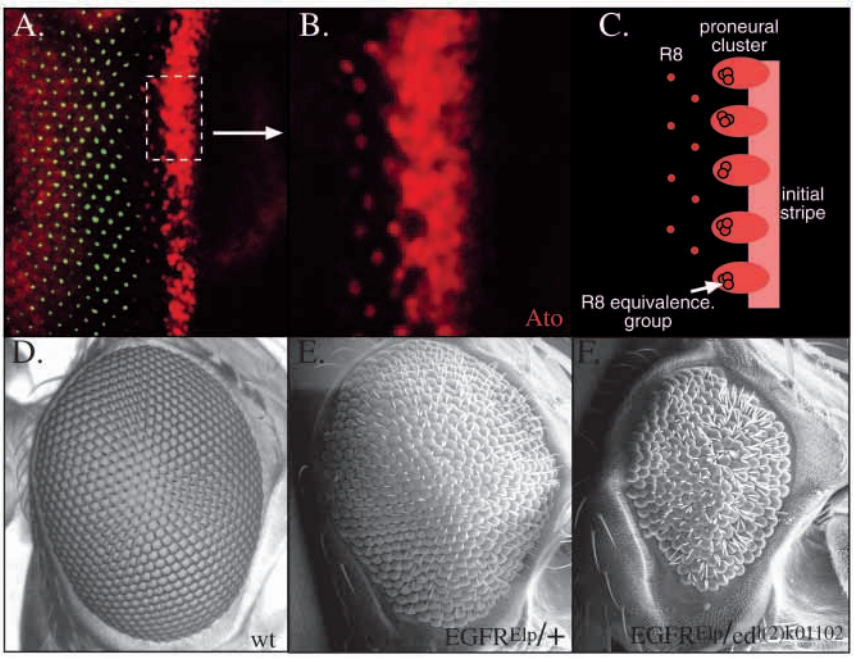

Fig. 1. Patterned expression of the bHLH transcription factor Atonal is essential for R8 formation in wild-type discs. (A) Atonal expression is patterned in the morphogenetic furrow of wild-type eye imaginal discs (red); terminally differentiated R8 cells express the protein Boss (green). (B,C) A close-up of Atonal immunostaining shows initial expression in proneural groups; the nuclei of two to three cells at the posterior edge of these groups rise apically, forming the R8 equivalence group. One cell from this group retains Atonal expression and differentiates as the R8 photoreceptor. (D) Scanning electron micrograph of a wild-type adult eye shows normal pattern formation. (E) $Egfr^{Ellipse}$ is an activated allele of Egfr; $Egfr^{Ellipse/+}$ flies have slightly smaller, mispatterned eyes. (F) Loss of one copy of *echinoid* in trans to $Egfr^{Ellipse}$ enhances the rough eye phenotype. Anterior is towards the left.

l(2)k01102 homozygotes are viable until late in the third larval instar (see Materials and Methods). Removal of the *l(2)k01102* P-element by standard excision protocol reverted the lethality and $Egfr^{Ellipse}$ interaction in 75% (18/24) of excision events and produced flies with no visible eye phenotype in either larvae or adults, indicating that the insertion was responsible for both the $Egfr^{Ellipse}$ interaction and the lethality. *l(2)k01102* contains an insertion in the 5′-untranslated region of the gene *echinoid*, a large transmembrane protein containing seven immunoglobulin repeats and a fibronectin III repeat on its extracellular face and a tyrosine-rich intracellular domain (Bai et al., 2001). Northern analysis confirmed that *l(2)k01102* is a null mutation; no *echinoid* transcript is detectable in *l(2)k00102* homozygous larvae (data not shown). Confirming observations by Bai et al. (Bai et al., 2001) *echinoid* mRNA and protein was found to be expressed ubiquitously throughout the eye disc (data not shown).

To examine the effect that loss of *echinoid* function had on R8 specification, we examined Atonal expression and R8 formation both in *echinoid* homozygotes and in eye discs mosaic for *echinoid*. In both cases, we found that R8 specification was disrupted. Atonal normally narrows from an equivalence group of two or three cells to a single cell posterior to the morphogenetic furrow. In genotypically *echinoid* discs, this narrowing fails to occur and Atonal remains present in small groups of cells as they emerge from the morphogenetic furrow (Fig. 2A,B). This defect is particularly conspicuous in young eye discs from early third instar animals in which the morphogenetic furrow has only advanced a short distance, although the defect is also widespread later in eye development and in clonal patches of cells lacking *echinoid* (Fig. 2C,D). We observed no defects, however, in the initial stages of Atonal expression, or in its resolution to proneural groups. We conclude from this that *echinoid* is required only for the resolution of the R8-equivalence group to a single cell.

The failure of Atonal expression to narrow to a single cell produces ommatidia that contain multiple mature R8 cells, as demonstrated with the R8-specific markers Boss (Fig. 2E-H) (Van Vactor et al., 1991) and Senseless (Fig. 2I,J) (Frankfort et al., 2001; Nolo et al., 2000). These R8s were commonly observed adjacent to one another; occasionally they were separated by another cell. This phenotype is similar to that of the mutations *rough* and *scabrous*, in which more than one cell of the two or three cell equivalence group differentiates as an R8. In nine third instar eye discs that were evaluated, 21% of ommatidia contained an extra R8 cell by Boss staining and 54% by Senseless staining. The disparity between the number of R8 cells detected by Boss and Senseless may reflect some difference in the specification of the extra R8 cells: Senseless is a direct transcriptional target of Atonal and begins expression within the morphogenetic furrow, while Boss typically begins expression 4-6 hours later, possibly reflecting an additional step in the differentiation of R8.

In adult fly eyes, the R8 photoreceptor is distinguished by a small inner rhabdomere, or light-gathering organ, in the bottom third of the retina. Although R7 cells also contain small rhabdomeres, these are visible only in more apical retinal sections. Consistent with the results in larval discs, genotypically *echinoid* tissue often contained multiple inner rhabdomeres in basal sections that did not extend through to more apical levels (Fig. 2K,L). R8 is the first cell to differentiate in the eye; it is required for the proper recruitment of the other photoreceptors and accessory cells that compose the mature ommatidium. The differentiation of more than one R8 cell within a single ommatidium disrupts the proper recruitment of other cell types; the result is often an enlarged or fused ommatidium containing more than the normal complement of eight photoreceptor neurons (Fig. 2K,L).

Our results contrast with those of a previous report where no defects in R8 specification were observed in *echinoid* mutants (Bai et al., 2001). Bai et al. report instead the presence of extra R7 cells in an *echinoid* background. We examined sections of adult eyes containing clonal patches of $ed^{l(2)k01102}$ tissue: in all clones where ommatidia had multiple R7 cells,

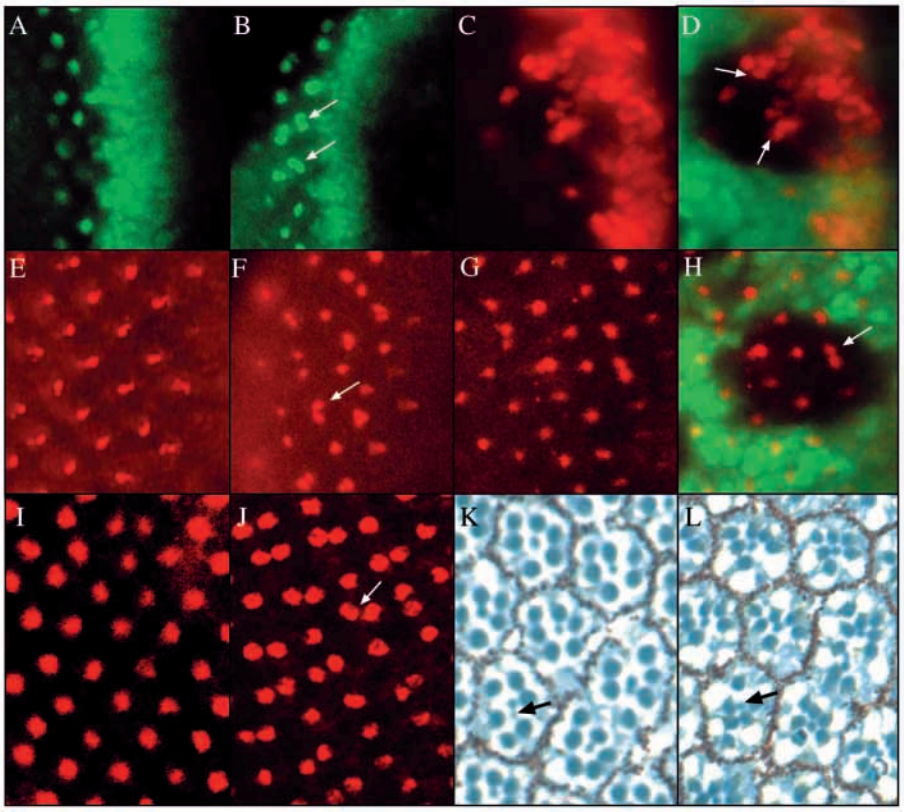

Fig. 2. Mutations in *echinoid* lead to formation of multiple R8 cells per ommatidium. (A) Atonal expression (green) narrows to a single cell posterior to the morphogenetic furrow in wild-type discs. (B) In homozygous $ed^{l(2)k01102}$ eye discs, multiple cells often retain Atonal expression (arrows). (C,D) Similarly, when $ed^{l(2)k01102}$ patches of tissue are created using the FLP-FRT technique (loss of green GFP marker), Atonal expression (red) within the patch fails to narrow to a single cell. (E) In wild-type tissue, one mature R8 photoreceptor is present in each ommatidium, as visualized with an antibody against Boss (red). (F) Multiple Boss-expressing cells are present in many ommatidia of $ed^{l(2)k01102}$discs. (G,H) Using the FLP-FRT technique to create homozygous $ed^{l(2)k01102}$ patches of tissue (loss of green) shows a similar multiple R8-phenotype by Boss staining (red). (I,J) In wild-type tissue (I), an antibody against Senseless detects one R8 photoreceptor per ommatidium, but in $ed^{l(2)k01102}/ed^{slA12}$ discs (J) many ommatidia contain multiple R8 cells (arrow). (K,L) In adult eyes, both R7 and R8 cells are distinguished by a small inner rhabdomere; the rhabdomere of the R7 cell is present in the apical portion of the eye, while the R8 rhabdomere is visible in more basal sections. Sections of adult eyes containing FLP-FRT-mediated clones of *echinoid* tissue show multiple inner rhabdomeres present in basal sections (arrow, K), but not necessarily in apical sections of the same ommatidia (arrow, L). Because the mutation used for these experiments is marked with the *white* gene, clonal boundaries cannot be discerned in these sections.

multiple R8 cells were present as well (n=11 clones). R8 induces the R7 fate (Van Vactor et al., 1991); our evidence, therefore, cannot distinguish whether these ectopic R7 cells are due solely to induction by ectopic R8 cells or are also a direct consequence of the *echinoid* mutation.

Loss of *echinoid* leads to sustained ERKA activation

One of the first hallmarks of R8 formation within the MF is the appearance of proneural clusters containing high levels of Atonal (Jarman et al., 1994; Sun et al., 1998). These groups of six to ten cells also contain high levels of ERKA phosphorylation (Kumar et al., 1998; Spencer et al., 1998) (Fig. 3A,B), a readout of Ras1-pathway signaling (Gabay et al., 1997). As the morphogenetic furrow progresses anteriorly, ERKA phosphorylation is normally lost and the proneural clusters resolve to single Atonal-expressing cells. By contrast, in *echinoid* discs a high level of ERKA phosphorylation was retained in small groups of cells even after the morphogenetic furrow had moved anteriorly (Fig. 3C-F).

Although Echinoid is expressed throughout the eye disc, the observed stabilization of phospho-ERKA is not due to a generalized upregulation of Ras1 pathway signaling throughout the disc. Not all cells display ERKA phosphorylation; rather, the patterned ERKA activation that was established in the morphogenetic furrow simply persists in cells of the proneural clusters for several rows. Because all ERKA phosphorylation within the morphogenetic furrow has been attributed to activation of Egfr (Wasserman et al., 2000), this suggests that the function of Echinoid in wild-type tissue is to downregulate Egfr signaling after a period of activation. Consistent with this, genetic interactions between Echinoid and the Egfr pathway have been shown in adult eyes (Bai et al., 2001) and in resolution of the R8 equivalence group to single cells (Rawlins et al., 2003).

echinoid and *argos* both restrict Egfr signaling during R8 development

Argos is another inhibitor of Egfr present in the eye disc during R8 formation. However, analysis of tissue lacking *argos* shows few defects in R8 patterning (Baonza et al., 2001; Freeman et al., 1992; Spencer et al., 1998), suggesting that another factor may be acting redundantly. To test whether Echinoid and Argos may be acting together to inhibit Egfr signaling in the morphogenetic furrow, we examined their ability to interact in vivo. Removing one copy of *argos* ($argos^{\Delta 7}$) mildly enhanced the $ed^{l(2)k01102}$ phenotype: the number of ommatidia with multiple R8 cells increased from 18% to 34%, as assessed with antibodies against Boss (n=412). The viable hypomorphic *argos* mutation, $argos^{W11}$, also enhanced the *echinoid* rough eye phenotype (Fig. 4A-D): the number of ommatidia with multiple R8s increased from 21% to 46% (n=336). This enhancement is presumably caused by a progressive increase in Egfr activity. However, Echinoid and Argos differ in their mode of action. Egfr inhibitors such as Argos and Kekkon are expressed in response to high levels of Egfr signaling to form negative feedback loops (Ghiglione et al., 1999; Schweitzer et

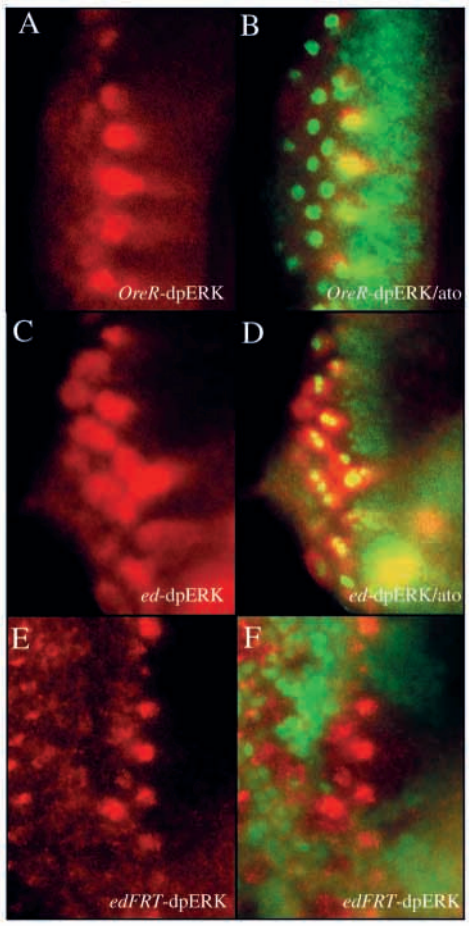

Fig. 3. Loss of *echinoid* leads to sustained ERKA phosphorylation. (A,B) High levels of phosphorylated ERKA, a readout of Egfr signaling, are present in a single row of cell clusters within the morphogenetic furrow of wild-type discs (anti-dpERK, red, A,B); these clusters correspond to the Atonal-expressing proneural clusters (anti-Atonal, green, B). (C,D) In homozygous $ed^{l(2)k01102}$ eye discs, these clusters fail to lose dpERK signaling (red) as cells emerge from the morphogenetic furrow and retain high levels of signaling for several rows. Groups containing high levels of dpERK retain Atonal expression in two or more cells (anti-Atonal, green). (E,F) Patches of $ed^{l(2)k01102}$ tissue created using the FLP-FRT technique (absence of green in F) retain high levels of phosphorylated ERKA relative to surrounding wild-type tissue (anti-dpERK in red).

al., 1995). Echinoid, by contrast, is ubiquitously expressed in the eye disc (Bai et al., 2001) (S.S. and R.C., unpublished), suggesting that it is not transcriptionally regulated by Egfr activity, which acts within discreet regions of the eye disc (Fig. 3A). Furthermore, unlike Argos, overexpression of Echinoid in the morphogenetic furrow does not lead to defects in ERKA phosphorylation or R8 specification (Fig. 4E-H). Our data indicate that specific Egfr signaling is not sensitive to relatively large changes in the amount of Echinoid present, and implies that if the activity of Echinoid is regulated, it is by a means other than transcription.

Echinoid can undergo homophilic interactions and is cleaved in S2 cells

The extracellular structure of immunoglobulin and fibronectin repeats in Echinoid suggests that it may be a member of the L1 class of cell-adhesion proteins, which are important in mediating axon guidance and cell fate decisions (reviewed by Kamiguchi and Lemmon, 2000; Walsh and Doherty, 1997). To examine the ability of Echinoid to mediate homophilic adhesive interactions, we transfected cultured S2 cells with epitope-tagged versions of Echinoid (see Materials and Methods). EdFLAG was capable of co-precipitating Edmyc (Fig. 5A), suggesting that Echinoid can form multimers. In the converse experiment, immunoprecipitation of Edmyc also co-precipitated EdFLAG (data not shown). A form of EdFLAG lacking extracellular sequences (EdFLAGΔN; see Fig. 5E)

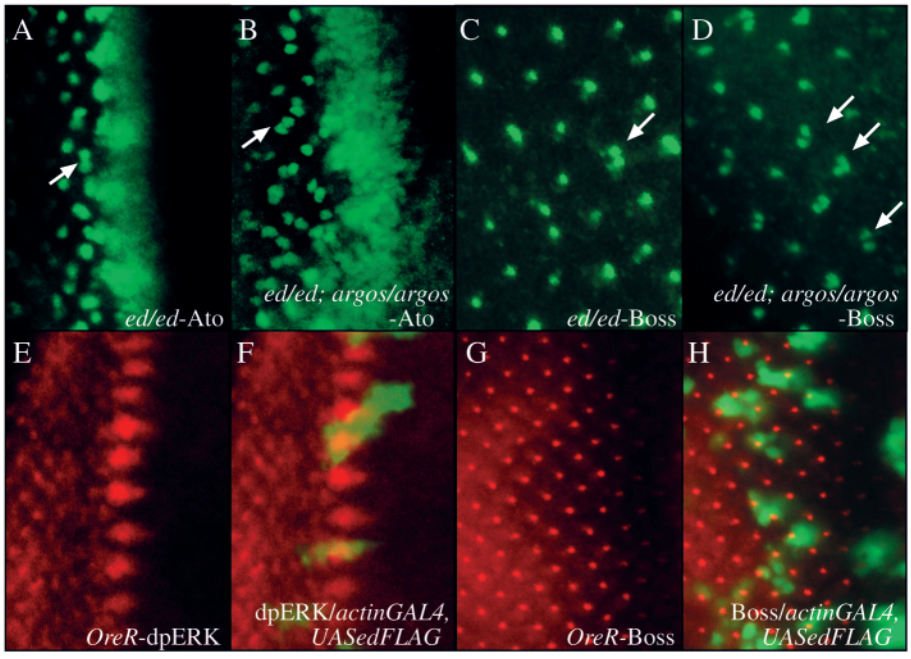

Fig. 4. *echinoid* and *argos* act in concert to inhibit Egfr signaling, but through different mechanisms. (A,B) Mutations in *argos* enhance the *echinoid* phenotype. (A) Discs homozygous for $ed^{l(2)k01102}$ show the multiple R8 phenotype when stained with anti-Atonal antibodies. An example of a pair of R8s is indicated by the arrow. (B) Mutations in *argos* ($argos^{W11}/argos^{W11}$)further enhance the $ed^{l(2)k01102}$ phenotype. (C,D) Same genotypes as in A,B. Enhancement of the multiple R8 phenotype is also apparent in more mature R8 cells as visualized with antibodies against Boss. (E-H) Echinoid-FLAG driven by the actin promoter was overexpressed in clonal patches of tissue using the FLP-out technique (green in F and H; see Materials and Methods). Unlike Argos, overexpression of Echinoid had no effect on the pattern of dpERK phosphorylation (E,F) or Boss expression (G,H).

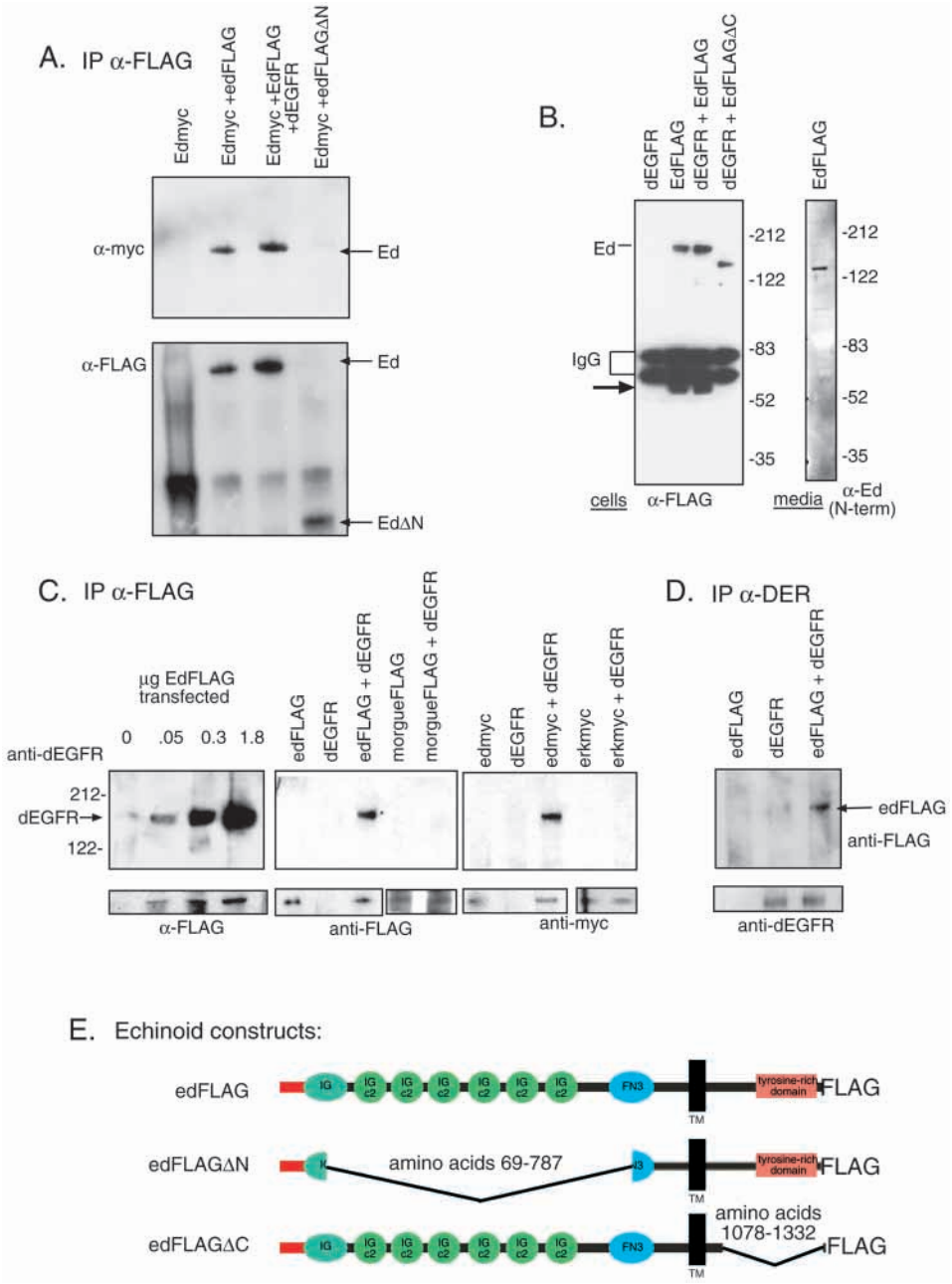

Fig. 5. Immunoprecipitation of Echinoid reveals homophilic interactions and interactions with Egfr. (A) Cultured S2 cells were transfected with EdFLAG and Edmyc to evaluate homophilic interactions. Immunoprecipitation with anti-FLAG Sepharose co-precipitates Edmyc; co-precipitation is not significantly affected by transfection with Egfr. (B) Analysis of immunoprecipitated Echinoid-FLAG (EdFLAG) revealed that a clipped form of Echinoid is also present, corresponding approximately to the 500 C-terminal amino acids (arrow, left blot). The amount of the clipped form is not altered by Egfr transfection. An antibody against the N-terminal region of Echinoid (Bai et al., 2001) detects a smaller form of Echinoid in media from these cells (right blot); this may represent shedding of the immunoglobulin and fibronectin III domains of Echinoid into the extracellular space. (C) Echinoid associates with Egfr. (Left panel) Cultured S2 cells were transfected with 1.8 μg Egfr DNA and variable amounts of EdFLAG DNA. Immunoprecipitation of EdFLAG co-precipitated increasing amounts of Egfr. (Middle and right panels) Cultured S2 cells were transfected with Egfr and EdFLAG or Edmyc DNA. Immunoprecipitation with FLAG-Sepharose or myc-Sepharose co-precipitated Egfr. Transfection of Egfr and other FLAG and myc-tagged proteins (morgueFLAG, erkmyc) did not lead to co-precipitation of Egfr.
(D) Immunoprecipitation of Egfr from cultured S2 cells transfected as in (C) co-precipitated EdFLAG. (E) Echinoid constructs used in these experiments: full-length Echinoid contains seven Ig repeats and a FN3 repeat on its extracellular face. EdFLAGΔN lacks the Ig repeats and the N-terminal part of the FN3 domain (amino acids 69-787) but not the signal sequence. EdFLAGΔC lacks the tyrosine-rich intracellular domain (amino acids 1078-1332).

did not co-precipitate Edmyc, suggesting that the proteins associate through their extracellular domains. Binding of EdFLAG to Edmyc was unaffected by transfection with Egfr. These results are consistent with the potential of Echinoid to act as a cell adhesion molecule; in keeping with this, defects in R8 differentiation have been observed in wild-type cells just outside *echinoid* mutant patches of tissue (Rawlins et al., 2003). However, the nature of this potential adhesive interaction on signaling is not clear, as overexpression of Echinoid, which might be expected to affect adhesion, had no effect on Egfr activity (see above).

Another common property of L1 immunoglobulin proteins is cleavage by plasmin or ADAM-class proteases at sites within or near FN3 domains (for a review, see Doherty et al., 2000). We observed a similar cleavage of Echinoid in S2 cultured cells. As shown in Fig. 5B, immunoprecipitations of C-terminally tagged Echinoid produced two bands on SDS-PAGE gels: one, which corresponds to full-length Echinoid, migrated as a 190 kDa protein (larger than the predicted molecular weight of 146 kDa, possibly owing to glycosylation); the other migrated at approximately 55 kDa, corresponding approximately to the C-terminal 500 amino acids of Echinoid. Consistent with this, an antibody to the N-terminal region of Echinoid detected a ~130 kDa form of the protein in media from transfected S2 cells. This suggests that, in addition to full-length Echinoid, cells also contain a truncated form which lacks most of the extracellular motifs, and that the Ig/Fibronectin domains of the protein are shed into the

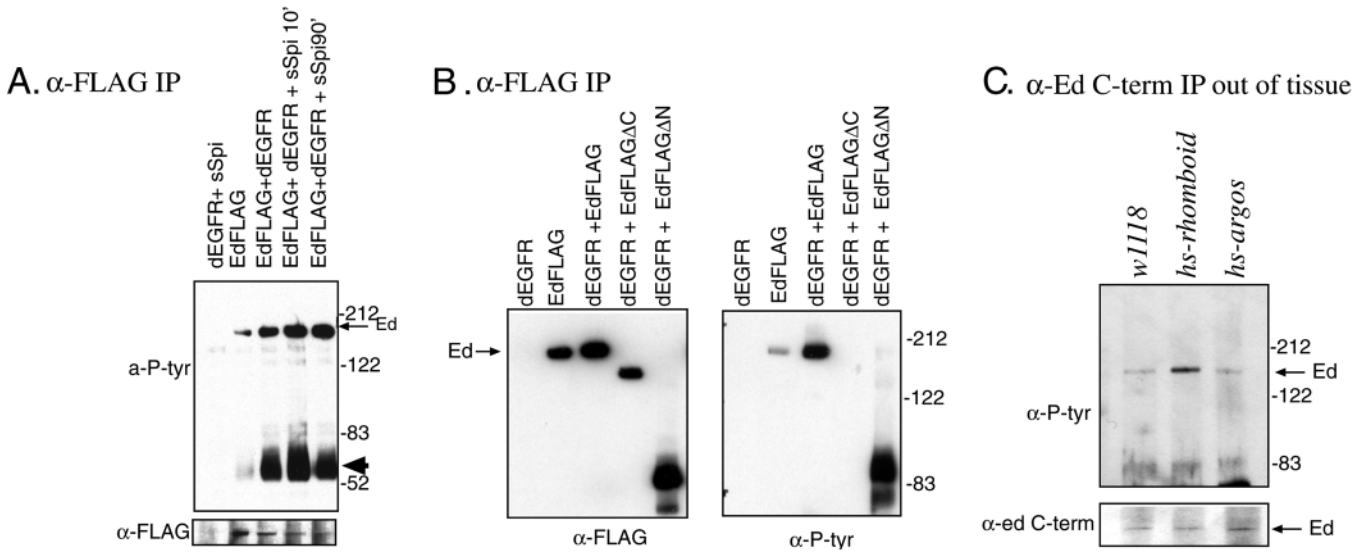

Fig. 6. Echinoid is phosphorylated in response to Egfr. (A) Immunoprecipitated EdFLAG from S2 cells transfected with EdFLAG and/or Egfr were analyzed by western blot using an antibody specific for phosphotyrosine. Echinoid was phosphorylated in response to Egfr transfection. This phosphorylation was further increased by addition of an active form of the Egfr ligand Spitz (sSpi) to the tissue culture medium. The clipped C-terminal form of Echinoid also becomes phosphorylated (arrow). (B) Echinoid lacking the intracellular domain (EdFLAGΔC) was not phosphorylated in response to Egfr signaling, but Echinoid lacking the extracellular motifs (EdFLAGΔN) was phosphorylated. (C) Echinoid immunoprecipitated from imaginal discs was also tyrosine-phosphorylated in response to Egfr signaling: Echinoid from w^{1118} imaginal discs shows a low-level of tyrosine phosphorylation. Activation of Egfr signaling by transient overexpression of Rhomboid increases phosphotyrosine associated with Echinoid; inhibition of Egfr by transient overexpression of Argos decreases phosphotyrosine.

surrounding extracellular space. The cleavage appears to be constitutive in S2 cells, and is not altered by transfection of Egfr (Fig. 5B).

Echinoid associates with Egfr

Echinoid has been proposed to act in a pathway parallel to Egfr, with signaling converging at the nucleus (Bai et al., 2001). Our data suggests that loss of *echinoid* acts upstream of the nucleus to stabilize ERKA phosphorylation. Given the localization of Echinoid at the cell surface (Bai et al., 2001) (data not shown) we explored the potential for direct interactions between Echinoid and Egfr in S2 cultured cells. FLAG-tagged Echinoid was immunoprecipitated and analyzed by western blots: transfection with increasing amounts of Echinoid co-precipitated increasing amounts of Egfr, suggesting that this is a specific interaction (Fig. 5C). Similarly, Echinoid containing a Myc tag also co-precipitated Egfr; other FLAG- and Myc-tagged proteins did not (Fig. 5C). In the converse experiment, immunoprecipitation of Egfr co-precipitated EdFLAG (Fig. 5D). The site of binding between Echinoid and Egfr could not be shown conclusively from these experiments: both EdFLAGΔC and EdFLAGΔN efficiently immunoprecipitated Egfr (data not shown), suggesting that their interaction occurs near the transmembrane domain or through multiple domains.

Echinoid is phosphorylated in response to Egfr activity

L1 cell-adhesion proteins are frequently regulated by phosphorylation, which controls their binding to other cytoplasmic signaling proteins. The physical association of Echinoid with Egfr, a receptor tyrosine kinase, suggested that it might be a substrate for tyrosine phosphorylation. To examine this possibility, we used S2 cell culture to assess tyrosine phosphorylation in response to Egfr signaling. As seen in Fig. 6A, Echinoid exhibited a low level of tyrosine phosphorylation in the absence of added Egfr. Co-transfection with Egfr, even without added ligand, led to a dramatic increase in the phosphorylation of Echinoid. This phosphorylation was increased further by addition of media containing a soluble form of the activating Egfr ligand Spitz. As seen in Fig. 6B, phosphorylation was limited to the tyrosine-rich intracellular region: a form of Echinoid lacking the intracellular domain (EdFLAGΔC; see Fig. 5E) was not phosphorylated. Interestingly, EdFLAGΔN, which lacks the extracellular immunoglobulin and fibronectin motifs, was phosphorylated in response to Egfr signaling, suggesting that these extracellular motifs are unnecessary for phosphorylation to occur. Bai et al. (Bai et al., 2001) suggested that Echinoid acts in a pathway parallel to Egfr, and that their activities converge in the nucleus. We find, however, that expression of the activated Ras isoform $dRas1^{Val12}$ did not lead to increased Echinoid phosphorylation (data not shown), indicating that phosphorylation of Echinoid in response to Egfr signaling occurs upstream of Ras activation. These data do not resolve whether Echinoid is phosphorylated directly by Egfr or by cytoplasmic tyrosine kinases immediately downstream of its activity, an issue also unresolved for other cell-adhesion proteins. Phosphorylation of Echinoid in response to Egfr signaling was confirmed in vivo: Echinoid immunoprecipitated from w^{1118} eye discs (which are essentially wild type at this stage) show a low level of phosphorylation (Fig. 6C). Echinoid immunoprecipitated from discs in which Rhomboid (an activator of Egfr) was transiently expressed shows an approximately fourfold increase in tyrosine phosphorylation. Transient expression of Argos, an

inhibitor of Egfr, produced either no change or a slight decrease in Echinoid phosphorylation levels over wild type. The importance of this phosphorylation to the function of Echinoid is not clear from these data. It has been noted, however, that strong overexpression of Echinoid in the developing retina leads to a rough eye in adults (Bai et al., 2001). This phenotype requires the presence of the Echinoid intracellular domain (Bai et al., 2001), suggesting that this domain may be essential for the function of Echinoid.

DISCUSSION

Egfr signaling is essential for the correct patterning and specification of all cell types in the *Drosophila* eye (Freeman, 1996). We find that loss of *echinoid* leads to stabilization of Egfr signaling and ERKA phosphorylation. As has been noted previously (Kumar et al., 1998; Spencer et al., 1998), activation of ERKA is closely correlated with expression of the R8 specification factor Atonal, and *echinoid* mutants show commensurate stabilization of Atonal expression, resulting in the formation of multiple R8 cells in many ommatidia. Mutations in *echinoid* and *Egfr* show strong mutual genetic interactions, suggesting that they influence R8 differentiation through a common pathway. Consistent with this view, we find that Echinoid and Egfr co-precipitate from cultured cells and that Echinoid is phosphorylated in response to Egfr signaling in vivo. These data suggest that Echinoid is required to downregulate Egfr signaling after a period of activation in order to limit the number of R8 cells, and may do so through direct interactions.

R8 patterning reflects at least two processes: spacing of emerging R8 equivalence groups and selection from these groups of single R8 cells. We previously suggested that expression of Egfr inhibitors is important for setting the spacing between R8 cells (Spencer et al., 1998), a view supported by mispatterning in loss-of-function Egfr clones (Dominguez and Freeman, 1998). We find, however, that *echinoid* plays no role in this process: while loss of *echinoid* does increase the duration of Egfr signaling, it does not affect the initial pattern of Egfr activity or the position of R8 equivalence groups within the morphogenetic furrow. Rather, Echinoid appears to be essential only for the second step in R8 specification, the selection of a single R8 cell from the 2-3 cell equivalence group. The role of Echinoid is to ensure that Egfr activity is downregulated within the group in a timely fashion; persistent Egfr activation appears to trigger all cells of the equivalence group to differentiate as R8s. Consistent with this, expression of an activated-Ras (Spencer et al., 1998), activated-Raf or Pointed-P1 (Rawlins et al., 2003) promote multiple R8 cells within individual ommatidia.

Interestingly, Echinoid is the second example of a co-factor required for fine-tuning a major signaling pathway during R8 selection. Selection of R8 from the equivalence group also requires *scabrous*, a modifier of Notch signaling (Cagan, 1993; Dokucu et al., 1996; Powell et al., 2001). Egfr and Notch signaling are used in a number of developing tissues. Echinoid and Scabrous appear to fill the need for high precision during resolution of the R8 equivalence group; this precision is almost unique in the developing nervous system. Therefore, Echinoid and Scabrous appear to have evolved to fine-tune these two pathways for the stringent requirements of the retina. We anticipate that other factors might provide similar fine-tuning to Egfr and Notch signaling in other tissues.

In the *echinoid* null allele described here ($ed^{l(2)k01102}$), only 54% of ommatidia contain multiple R8s (fewer by Boss staining), suggesting that another factor may be acting redundantly to downregulate Egfr signaling in some cells. One candidate for a redundant factor is a highly homologous gene distal to *echinoid* on the second chromosome. Preliminary data indicates that this gene, which we refer to as *fred* (*friend of echinoid*), is expressed in the same tissues as *echinoid* and displays similar interactions with $Egfr^{Ellipse}$ (S.S., unpublished) (The FlyBase Consortium, 2003). Further examination of the *fred* phenotype and creation of *fred ed* lines will be necessary to determine if *fred* acts in a manner similar to *echinoid*.

In its extracellular domain, Echinoid appears similar to other members of the L1 family of proteins: it undergoes homophilic binding and ectodomain shedding, presumably to regulate cell-cell adhesion. Although some L1 cell adhesion proteins have been shown to interact with receptor tyrosine kinases such as Egfr, those that have been described to date lead to activation, not inhibition, of MAP kinase phosphorylation (Schaefer et al., 1999; Thelen et al., 2002). In addition, Echinoid lacks two intracellular motifs common to many L1 proteins: a clathrin sorting motif (YRSLE), which regulates internalization, and an ankyrin-binding domain (NEDGSFIGQY), which controls association with the cytoskeleton, suggesting that Echinoid acts by a different mechanism from other L1 proteins. As we find that overexpression of Echinoid in tissue has no effect on the level of phosphorylated MAP kinase, a read-out of Egfr signaling, it appears that Echinoid does not act as a general inhibitor of Egfr. Instead, the prolonged presence of phosphorylated MAP kinase in *echinoid* mutants suggests that the role of Echinoid is to downregulate Egfr signaling after a period of activation. Below, we explore possible (and not mutually exclusive) models for the function of Echinoid.

The ability of Egfr to signal depends on its localization and its downstream targets. Ligand-induced endocytosis is a well-documented mechanism for downregulating Egfr activity (for a review, see Carpenter, 2000), and the prolonged Egfr signaling we observe in *echinoid* mutants suggests that one possible role for Echinoid is to facilitate Egfr endocytosis after a period of activity. Another notable feature of Echinoid is its unusual intracellular domain, which differs from other members of the L1 superfamily. This domain is likely required for at least some aspects of Echinoid function (Bai et al., 2001) (see above), and suggests that Echinoid may target downstream signaling molecules. Based on our results, this unknown pathway would intersect with Egfr signaling prior to MAPK phosphorylation.

What downstream molecules might be targeted by Echinoid? One potential model for the function of Echinoid is provided by work on the vertebrate SIRPα proteins, the only group of Ig-containing proteins shown to inhibit receptor-tyrosine kinase (RTK) signaling (Kharitonenkov, 1997) (reviewed by Cant and Ullrich, 2001; Vely and Vivier, 1997). SIRP-α proteins are phosphorylated on tyrosine in response to RTK activation; these phosphorylated residues provide binding sites for the SHP2 tyrosine phosphatase. Analysis of the *Drosophila* genomic sequence uncovered no clear *Drosophila* orthologs of SIRP-α proteins, but the overall structural similarity of

Echinoid, its phosphorylation in response to dEGFR signaling and its importance in downregulating dEGFR signaling suggest that it may function in a manner analogous to the SIRP-α proteins. We have observed genetic interactions between *echinoid* and *corkscrew*, the *Drosophila* homolog of SHP2, and have been able to detect binding between these proteins in cultured cells (S.S., unpublished). However, the significance of these interactions will require further study in vivo.

We gratefully acknowledge N. Baker, R. Herbst, J.-C. Hsu, E. Perkins, B.-Z. Shilo, I. Rebay, H. Okano and M. Simon for providing reagents, A. Jarman and E. Rawlins for generously sharing unpublished results and reagents, and the Cagan laboratory for shared reagents and helpful comments. This work was supported by National Science Foundation Grant IBN-9604090 and National Institutes of Health R01CA84309.

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