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Neuroglian activates Echinoid to antagonize the *Drosophila* EGF receptor signaling pathway

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SUMMARY

***echinoid* (*ed*) encodes an cell-adhesion molecule (CAM) that contains immunoglobulin domains and regulates the EGFR signaling pathway during *Drosophila* eye development. Based on our previous genetic mosaic and epistatic analysis, we proposed that Ed, via homotypic interactions, activates a novel, as yet unknown pathway that antagonizes EGFR signaling. In this report, we demonstrate that Ed functions as a homophilic adhesion molecule and also engages in a heterophilic trans-interaction with *Drosophila* Neuroglian (Nrg), an L1-type CAM. Co-expression of *ed* and *nrg* in the eye exhibits a strong genetic synergy in inhibiting EGFR signaling. This synergistic effect requires the intracellular domain of Ed, but not that of Nrg. In addition, Ed and Nrg colocalize in the *Drosophila* eye and are efficiently co-immunoprecipitated. Together, our results suggest a model in which Nrg acts as a heterophilic ligand and activator of Ed, which in turn antagonizes EGFR signaling.**

Key words: EGF receptor, Cell adhesion, Echinoid, Neuroglian, Signaling, *Drosophila*

INTRODUCTION

Members of the immunoglobulin (Ig) superfamily represent one of the three major classical groups of cell-adhesion molecules (CAMs) and play important roles in developmental processes in all vertebrate and invertebrate species. CAMs do not simply provide a specific adhesiveness between cells and to the extracellular matrix, but they also play active roles in modulating cell-cell communication and signaling. Ig domain CAMs specifically interact with several receptor tyrosine kinase (RTK) systems, including the epidermal growth factor receptor (EGFR) and the fibroblast growth factor receptor (FGFR) pathways (Garcia-Alonso et al., 2000; Williams et al., 1994). Apparently, cell adhesion and signaling are closely linked processes.

The *Drosophila* eye is composed of about 800 ommatidia, each of which includes eight light-sensing photoreceptor neurons (R1-R8), four non-neuronal cone cells and eight accessory cells. EGFR activity is required for the differentiation of all cell types, with the exception of photoreceptor R8 cells (Dominguez et al., 1998; Freeman, 1996). In this study, we have used the development of the *Drosophila* compound eye as the experimental paradigm to explore the physical and functional interactions between two *Drosophila* Ig-domain containing CAMs, Echinoid (Ed) and Neuroglian (Nrg), and their effect on EGFR signaling. The Ig superfamily is well represented in the *Drosophila* genome, which contains about 150 genes encoding Ig domain proteins. Many of these gene products have cell adhesion functions and fulfill important roles during *Drosophila* development (Hynes and Zhao, 2000). Ed has seven Ig domains, two fibronectin type III (Fn III) domains and a transmembrane (TM) domain, followed by a 315 amino acid intracellular domain with no identifiable structural or functional amino acid motif (Bai et al., 2001). *ed* was originally isolated as an enhancer of the rough eye phenotype caused by Elp^{B1}, a gain-of-function *Egfr* allele, and genetically interacts with several components in the EGFR pathway. As a consequence, the *ed* mutant phenotype includes the generation of extra photoreceptor and cone cells in the *Drosophila* eye. Conversely, overexpression of *ed* in the eye leads to a reduction of photoreceptor cell number. These results indicate that *ed* is a negative regulator of the EGFR pathway. Based on genetic mosaic and epistatic analyses, we proposed that Ed acts as a homotypic cell adhesion protein, which antagonizes EGFR signaling by regulating the activity of the TTK88 transcriptional repressor, the most downstream component of the EGFR signaling pathway (Bai et al., 2001).

L1-type proteins comprise six Ig domains, three to five Fn III repeats and a cytoplasmic domain with a conserved ankyrin-binding site. This family of transmembrane proteins includes L1-CAMs, neurofascins, NrCAMs, NgCAM and CHL1s, in vertebrates, and Neuroglians, in invertebrate species (for a review, see Brümmendorf et al., 1998; Hortsch, 2000). During nervous system development, L1-type CAMs

have been implicated in neurite outgrowth, axon guidance and neurite fasciculation, which employ both homophilic and heterophilic interactions (for a review, see Crossin and Krushel, 2000; Hortsch, 2000). Mutations in the human *L1CAM* gene result in mental retardation and other neurological phenotypes, for which summarily the term CRASH syndrome has been coined. This emphasizes the importance of *L1CAM* for the development of the nervous system (Kamiguchi et al., 1998). Nrg is the *Drosophila* homolog of the vertebrate L1 family proteins (Bieber et al., 1989). Alternative splicing of the primary transcript produces two protein isoforms of Nrg, which differ in their intracellular domain (Hortsch et al., 1990). The Nrg^{180} isoform is neuron specific, whereas the Nrg^{167} isoform is expressed more broadly. The presence of a highly conserved FIGQY ankyrin-binding site enables Nrg and other L1-type proteins to assemble membrane skeleton components at sites of cell-cell contact (Dubreuil et al., 1996; Hortsch et al., 1998). Phosphorylation of the tyrosine residue in the FIGQY motif abolishes this ankyrin-binding activity (Garver et al., 1997; Tuvia et al., 1997). An analysis of *Drosophila* lines with homozygous lethal mutations in the *nrg* gene demonstrated alterations in motoneuron axon pathfinding and other neurological phenotypes (Hall and Bieber, 1997). In addition, Nrg autonomously increases the activity of both the EGFR and FGFR to control growth cone decisions (Garcia-Alonso et al., 2000) (R.I. and M.H., unpublished). Therefore, both Ed and Nrg are involved in the regulation of RTK signaling processes.

In this paper, we have used a genetic co-expression screen to identify Nrg as a non-autonomous ligand of Ed. When compared with overexpression of either *ed* or *nrg* alone, co-expression of both molecules together uncovers a specific synergistic effect in inhibiting EGFR signaling. By using a S2 cell expression system we also demonstrate a direct heterophilic trans-interaction between Ed and Nrg. The observation that only the intracellular domain of Ed is required for the EGFR signal repression leads us to propose that Ed is the signal-receiving molecule and is activated by either its own homophilic interaction or by Nrg. Subsequently, it autonomously represses the EGFR signaling pathway by a so far unknown mechanism.

MATERIALS AND METHODS

Drosophila stocks

The stocks used in this study were the *nrg* protein null mutation nrg^{1} (Hall and Bieber, 1997); *UAS-ed* and $UAS\text{-}ed^{intra}$ (Bai et al., 2001). The $UAS\text{-}nrg^{180}$, $UAS\text{-}nrg^{167}$ and the $UAS\text{-}nrg^{GPI}$ constructs were made by subcloning previously characterized Nrg cDNAs into the pUAST vector (Hortsch et al., 1995). Transgenic lines were generated by P-element-mediated transformation (Spradling and Rubin, 1982).

Molecular biology

$UAS\text{-}ed^{C50}$ was generated by ligating two overlapping PCR products, with the first PCR product containing the TM domain plus the following 22 amino acids of Ed and a second PCR product containing the last C-terminal 50 amino acids of Ed. For the S2 cell expression studies, cDNAs were subcloned into the pRmHa3 vector under the control of the *Drosophila* metallothioneine promoter (Bunch et al., 1988). The constructs for *Drosophila* $Nrg^{180}$and Nrg^{GPI} have been described previously (Hortsch et al., 1995). The pRmHa3-*Drosophila* Fasciclin 1 plasmid was constructed by ligating a 3.0 kb *Eco*RI Fasciclin 1 cDNA fragment into the *Eco*RI restriction site of the pRmHa3 vector. The Echinoid cDNA was subcloned into pRmHa3 as a 4.3 kb *Sma*I/*Sal*I fragment and subsequently modified by the removal of a 3′ 0.5 kb *Nsi*I/*Sal*I fragment, which was replaced by an oligonucleotide cassette encoding the HA-epitope (Kolodziej and Young, 1991). This substitutes the 73 C-terminal amino acid residues of the natural Ed protein with two copies of the HA-epitope (YPYDVPDYA).

Histology

Scanning electron micrographs were prepared as described (Kimmel et al., 1990). Immunohistochemical staining of imaginal discs was performed as described (Bai et al., 2001)using anti-ELAV (rat, 1:250, Developmental Studies Hybridoma Bank), anti-Cut (mouse, 1:5, Developmental Studies Hybridoma Bank), anti-Ed (rabbit, 1:200) (Bai et al., 2001) and 1B7 (mouse, 1:200) (Bieber et al., 1989). Cy3- and Cy5-conjugated secondary IgGs are from Jackson ImmunoResearch Laboratories. Confocal microscopy was performed using Zeiss Model Pascal.

Transfection and maintenance of S2 cells

Schneider 2 (S2) cells were maintained at 25°C in Schneider's medium with 10% fetal calf serum and penicillin/streptomycin (all reagents were from Life Technologies). S2 cells were transfected with Lipofectin Reagent (Life Technologies) according to the manufacturer's protocol and transfected cells were selected using hygromycin resistance (250 μg/ml hygromycin B; Sigma), which was conferred by the pCO_{hygro} plasmid (Invitrogen). A detailed protocol for establishing cloned S2 cell lines using soft agar cloning has been reported previously (Bieber, 1994). Individual cell clones were induced overnight with 0.7 mM $CuSO_4$ and analyzed by Western blotting for high expression of the transfected cDNAs. Selected lines, designated S2:Ed, $S2:Nrg^{180}$, $S2:Nrg^{GPI}$ or S2:Fas1, expressed either the HA-epitope-tagged form of *Drosophila* Echinoid, the neuronal or the artificial GPI-anchored isoform of *Drosophila* Neuroglian or *Drosophila* Fasciclin 1, respectively.

Cell aggregation assays

Usually 2×10^6 cells were labeled using the Cell-Tracker™ CM-DiI reagent (Molecular Probes) for 2 hours at 25°C in serum-free medium. The labeled cells were washed with complete S2 cell medium five times to remove excess dye and induced overnight with 0.7 mM $CuSO_4$. Labeled, induced cells were mixed with unlabeled, induced cells as indicated to a final concentration of 1.5×10^6 cells/ml and incubated on a rotating shaker at 200 rpm for 2 hours at room temperature. Small aliquots of aggregated cells were mounted on microscope slides under a #2 coverslip bridge and examined and photographed using a Nikon Optiphot 2 microscope (Nikon), which was equipped with Nomarski and rhodamine channel epifluorescence optics and a Nikon DXM1200 digital camera.

SDS-polyacrylamide gel electrophoresis and western blot analysis

Induced S2 cells were collected by centrifugation (2.5×10^5 cells/lane) and solubilized using SDS-polyacrylamide sample buffer. Solubilized S2 cell proteins or immunoprecipitated proteins were separated by electrophoresis in 10% SDS-polyacrylamide gels and transferred onto nitrocellulose filters. Subsequently, the blots were probed with specific primary and with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) and developed with 3,3′-diaminobenzidine as described by Hortsch et al. (Hortsch et al., 1985). The HA.11 monoclonal antibody was a gift from Dr R. Dubreuil (University of Illinois at Chicago). The 1B7, BP-104 and the 3C1 mouse monoclonal antibodies against *Drosophila* Neuroglian have been described and characterized previously (Bieber et al., 1989; Hortsch et al., 1990; Hortsch et al., 1995).

Co-immunoprecipitation procedure

Immunoprecipitations were performed using a modification of the protocol by Anderson and Blobel (Anderson and Blobel, 1983). Transfected cells were induced overnight with 0.7 mM $CuSO_4$ and mixed and incubated on a shaking platform as indicated. A total of 10×10^6 cells were collected by centrifugation for each immunoprecipitation and solubilized in a buffer containing 1.25% Trition X-100. The soluble fraction was incubated overnight at 4°C with either 1B7 anti-Nrg or with a control monoclonal antibody. 50 μl of a 1:1 Protein G-Sepharose suspension (Amersham Pharmacia Biotech) was added and incubated at room temperature on a rotator for 2 hours. The Protein G-Sepharose beads were collected by centrifugation and washed four times. Bound proteins were eluted with SDS-polyacrylamide gel electrophoresis sample buffer and probed after SDS-polyacrylamide gel electrophoresis by western blot analysis.

RESULTS

Overexpression of *ed* and *nrg* results in a loss of photoreceptor and cone cells

As EGFR activity is required for the differentiation of both photoreceptor (except R8) and cone cells, we used the numbers of these cell types per ommatidum as a readout for EGFR activity in the eye disc. Flies with a mutation in the *ed* gene produce extra photoreceptor and cone cells. By contrast, overexpression of *ed* in the eye leads to a reduction of the number of photoreceptor cells per ommatidium. These findings together with additional genetic evidence indicated that Ed uses an independent pathway to antagonize EGFR signaling, and we postulated that this inhibition might be initiated by a homophilic binding activity of Ed (Bai et al., 2001). To explore the possibility that Ed could be involved in heterophilic interactions with other Ig domain CAMs, we conducted a genetic overexpression screen. We reasoned that if *ed* acts as a heterophilic receptor, overexpression of both the Ed receptor and its potential ligand(s) should have a synergistic effect on the inhibition of EGFR signaling, which results in a reduced number of cone and photoreceptor cells. In addition, both adhesion molecules must normally be co-expressed and colocalized in the developing eye disc in order to engage in a functional heterophilic adhesive interaction.

We used the GMR-GAL4 driver line to co-express *UAS-ed* with several available UAS and EP lines that drive overexpression of various Ig domain-containing adhesion molecules. As shown previously, ectopic expression of Ed in the eye results in a rough eye phenotype and a loss of photoreceptor and cone cells (Bai et al., 2001) (Fig. 1B,F,J). On average, 10-15% of ommatidia were missing photoreceptor or cone cells (Table 1). By contrast, overexpression of either the neuronal nrg^{180} or the non-neuronal nrg^{167} isoform alone had no effect on the number of photoreceptor or cone cells (Fig. 1C,G,K; Table 1). However, co-expression of both *ed* and nrg^{180} (or nrg^{167}) resulted in a more severe rough eye phenotype (Fig. 1D) with a reduction of the number of ommatidia, a varying size of ommatidia and a decrease in the number of bristles. In addition, a significantly higher percentage of ommatidia contained fewer photoreceptor and cone cells (Fig. 1H,L; Table 1). No synergistic effects were detected when *ed* was overexpressed together with other CAMs, such as *Drosophila* Fasciclin 2 or human L1CAM (data not shown).

To document the interaction between Ed and Nrg further, we examined the effect of overexpression of *ed* in female flies, which had only one copy of the *nrg* gene. nrg^1 is a *nrg* null allele (Hall and Bieber, 1997). A reduction in half of the

Table 1. *ed* genetically interacts with *nrg*

Genotype	Photoreceptor loss (%)	Cone cell loss (%)
GMR>ed	16.2 (*n*=198)	12.1 (*n*=993)
GMR>Nrg^{180}	0.0 (*n*=130)	0.0 (*n*=150)
GMR>Nrg^{GPI}	0.0 (*n*=108)	0.0 (*n*=145)
GMR>ed + Nrg^{180}	50.7 (*n*=223)	69.0 (*n*=423)
GMR>ed + Nrg^{GPI}	83.8 (*n*=370)	71.9 (*n*=178)
GMR>ed^{C50}	31.1 (*n*=160)	26.3 (*n*=137)
nrg^1*/+; GMR>ed*	15.8 (*n*=476)	9.5 (*n*=1550)

The numbers represent the percentage of ommatidia in midpupal eye discs, which contained fewer than seven Elav-positive photoreceptor and fewer than four Cut-positive cone cells, respectively. *n*, the total number of ommatidia that were evaluated. The enhancement of the *GMR>ed* overexpression phenotype by *GMR>Nrg* is highly significant ($P<0.0001$; χ^2 test).

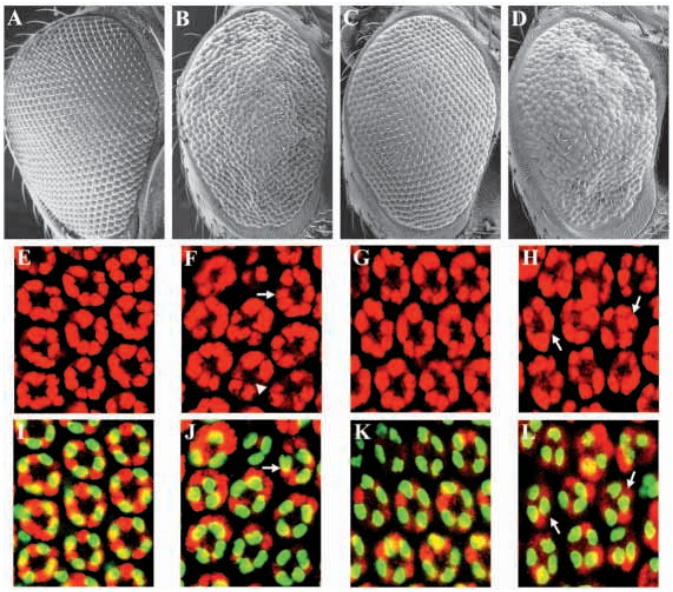

Fig. 1. Overexpression of Ed and Nrg results in loss of photoreceptor and cone cells. Scanning electron micrographs (A-D) of adult eyes, and midpupal eye imaginal discs stained for the photoreceptor marker ELAV (red, E-H) and cone cell marker Cut (green, I-L). (A,E,I) Wild type; (B,F,J) *GMR-GAL4/UAS-ed*; (C,G,K) *GMR-GAL4/UAS-*nrg^{180}; (D,H,L) *GMR-GAL4/UAS-ed/UAS-*nrg^{180}. (I-L) The upper focal planes of E-H, respectively. There are seven photoreceptors (E, R8 is out of the plane of focus) and four overlying cone cells (I) in wild-type imaginal discs. Overexpression of *UAS-ed* in eye causes a mild rough eye (B), ommatida with six or fewer photoreceptor neurons (arrowhead in F), and ommatidia with three or fewer cone cells (J). The arrows in F and J indicate the same ommatidium that contains seven photoreceptor (F) and three cone cells (J). Overexpression of nrg^{180} (C,G,K) alone causes no phenotype at 25°C. However, co-expression of both *ed* and nrg^{180} results in a more severe rough eye phenotype (D), as manifested by reduced number of ommatidia, varying size of ommatidia and decreased number of bristles. In addition, a much higher percentage of ommatidia contain fewer photoreceptor (arrows in H) and cone cells (arrows in L).

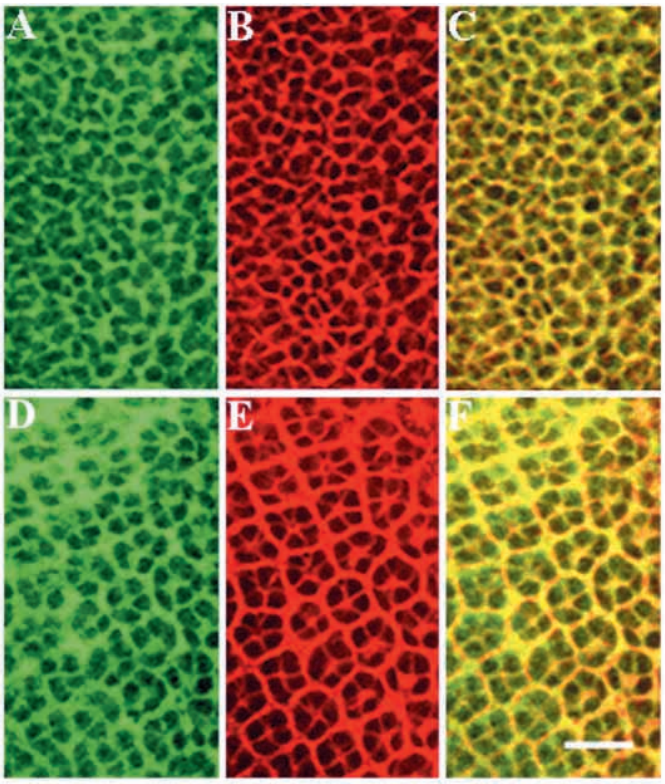

Fig. 2. Ed is colocalized with Nrg. Third instar larval eye discs were double-labeled with anti-Ed antibodies (green) and anti-Nrg monoclonal antibody 1B7 (red). Both Ed and Nrg are co-expressed in the basal undifferentiated cells (A-C) and in developing photoreceptor clusters (D-F). Scale bar: 10 μm.

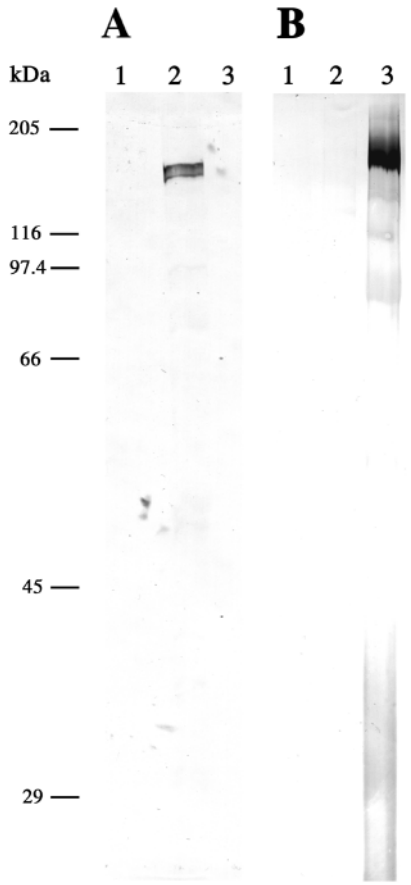

Fig. 3. Western blot analysis of Ed and Nrg protein expressed by transfected S2 cell lines. Shown is a western blot analysis of induced, native (lane 1) and transfected S2 cells. Each lane contains the total protein from 2.5×10^5 cells. Lane 2 represents cells that were transfected with a construct encoding the neuronal isoform of Nrg, whereas lane 3 contains cells that were transfected with a HA epitope-tagged version of *Drosophila* Ed. Blot A was incubated with the anti-Nrg monoclonal antibody BP-104 and blot B with the HA.11 monoclonal antibody, respectively.

nrg gene dosage significantly suppresses the cone cell loss phenotype ($P<0.0369$), but not the loss of photoreceptor cells ($P=0.8957$), which are both caused by GMR-GAL4-driven *UAS-ed* expression (Table 1). Together, these results demonstrate a specific genetic interaction between *ed* and both protein isoforms of *nrg*.

In the developing *Drosophila* imaginal eye disc Ed is colocalized with Nrg

The genetic synergy between Ed and Nrg suggests that both proteins might also physically interact with each other. Using antibodies that specifically recognize Ed and both isoforms of Nrg for an immunocytochemical analysis, we first tested this possibility by examining their expression pattern in the developing *Drosophila* eye disc. Both Ed and Nrg are colocalized to all cells throughout the third instar larval eye disc, including undifferentiated cells (Fig. 2A-C) and developing ommatidial clusters (Fig. 2D-F).

Ed acts as a homophilic adhesion protein

Similar to several vertebrate Ig-domain CAMs, which interact with members of the L1 family, Ed might exhibit both homo-, as well as heterophilic adhesive activities. Our previous genetic results and the data presented above both support this possibility. To investigate the adhesive function of Ed, we expressed *Drosophila* Ed protein in *Drosophila* Schneider 2 (S2) cells. Owing to their lack of endogenous CAMs, S2 cells have been successfully used for the functional analysis of a range of adhesive proteins (Hortsch and Bieber, 1991). As demonstrated by the western blot in Fig. 3B, HA epitope-tagged Ed protein is expressed by S2 cells at a high and stable level. This epitope-tagged version of the Ed protein exhibits an apparent molecular weight of about 160 kDa, about the size to be expected from the Ed amino acid sequence. When S2 cells, which express Ed protein, were allowed to aggregate, they formed small to medium-sized cell clusters. In order to establish whether this cell aggregation was due to a homophilic activity of Ed or to a heterophilic interaction with an endogenous ligand, which might be expressed on the S2 cell surface, cell-mixing experiments were performed (Fig. 4). Aggregation experiments, in which unlabeled, Ed-expressing cells were mixed with DiI-labeled, Ed-expressing cells, resulted in mixed cell clusters, consisting of labeled and unlabeled cells (Fig. 4A,B). By contrast, DiI-labeled, native S2 cells, which do not express Ed protein, were not recruited into Ed cell aggregates (Fig. 4C,D). These results demonstrate that Ed acts as a homophilic adhesion protein and

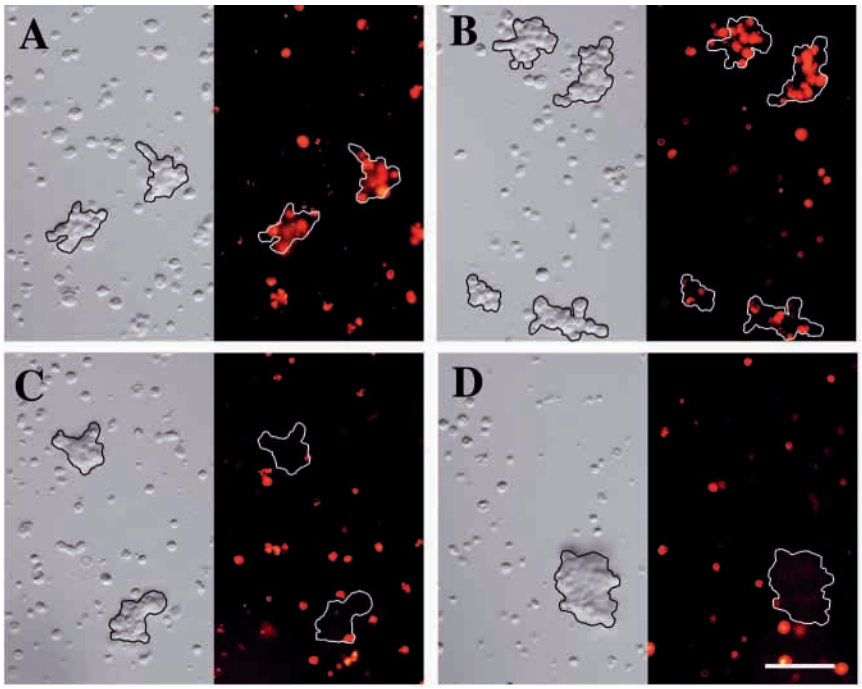

Fig. 4. Ed protein induces homophilic cell aggregation of S2 cells. Shown are the results of a S2 cell mixing experiment, in which unlabeled and DiI-labeled S2 cells were mixed at a ratio of 1: 1 and co-aggregated. (A,B) Two views of areas with labeled and unlabeled S2 cells, which are both expressing Ed protein. The left part of each panel depicts the view using Nomarski optics, whereas the same area is shown in the right part of the panel using epifluorescence with a rhodamine filter set. In C,D, unlabeled Ed-expressing S2 cells were co-aggregated with DiI-labeled, native S2 cells. Scale bar: 70 μm.

exhibits no heterophilic affinity to any endogenous S2 cell protein.

Ed and Nrg engage in a robust heterophilic trans-interaction

Similar mixing experiments as described in the previous paragraph were performed to determine whether Nrg and Ed engage in a heterophilic interaction (Fig. 5). In comparison with Ed-expressing S2 cells, S2 cells expressing Nrg exhibit a much stronger homophilic cell adhesion capability, resulting in very large S2 cell aggregates. The co-aggregation of Ed-expressing cells with Nrg-expressing S2 cells yielded mixed cell aggregates, consisting of cells from both cell populations (Fig. 5A). As shown previously (Hortsch et al., 1995), DiI-labeled, native S2 cells are not incorporated into Nrg-cell clusters (Fig. 5B). Fig. 5C,D and the quantitative evaluation shown in Table 2 demonstrate that the heterophilic interaction of Ed is specific to S2 cells expressing Nrg and that Ed does not interact with other *Drosophila* adhesion molecules, such as *Drosophila* Fasciclin 1 (Elkins et al., 1990). A mixture of S2 cells, which expressed either Ed or Fasciclin 1, resulted in two separate types of cell clusters, which were either unlabeled or completely DiI-labeled (Fig. 5C). Thus, Ed and Nrg engage in a robust and specific heterophilic trans-interaction.

Ed is co-immunoprecipitated with Nrg

The strength and stability of this interaction was demonstrated by co-immunoprecipitation experiments. As native Nrg interacts with ankyrin and the S2 cell membrane skeleton, it becomes resistant to non-ionic detergent extraction after engaging in cell adhesion (Dubreuil et al., 1996). Therefore, an

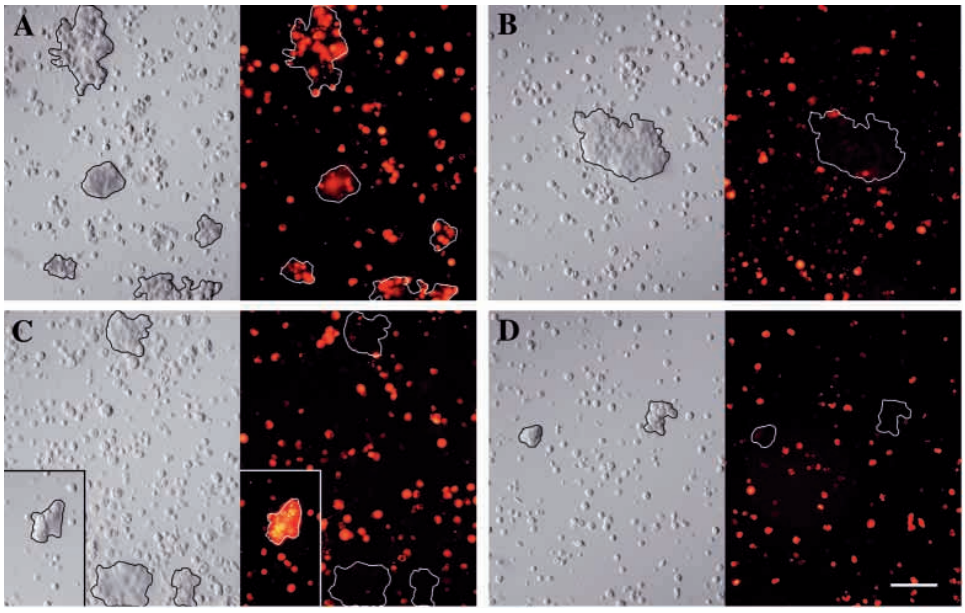

Fig. 5. Ed and Nrg protein are heterophilic binding partners, when expressed on separate populations of transfected S2 cells. Shown are representative areas of four combinations of S2 cell mixtures, which have been co-aggregated. The left sides of all panels show areas of S2 cells using Nomarski optics, with the same areas shown on the right side using epifluorescence, thereby visualizing the DiI-labeled cells. For all experiments, DiI-labeled cells and unlabeled cells were mixed at a ration of 1: 1 and subsequently aggregated on a shaking platform. (A) A mixture of DiI-labeled, Ed-expressing cells with unlabeled, Nrg-expressing S2 cells; (B) DiI-labeled, native S2 cells with unlabeled, Nrg-expressing cells; (C) DiI-labeled, Ed-expressing cells with unlabeled, Fasciclin 1-expressing cells; (D) DiI-labeled, native S2 cells with unlabeled, Fasciclin 1-expressing S2 cells. The inserts in C depict a small aggregate of S2 cells, which consists entirely of DiI-labeled and therefore Ed-expressing cells. Scale bar: 70 μm.

Table 2. S2 cell mixing experiments demonstrate that Ed engages in heterophilic interactions with Neuroglian and also is a homophilic CAM

Unlabeled cells	Labeled cells	% of cell aggregates with DiI-labeled cells
Nrg^{180}	S2	3.3±0.6
Nrg^{180}	Ed	97.0±0.8
Nrg^{180}	Fas1	4.0±1.0*
Fas1	S2	2.7±0.6
Fas1	Ed	3.3±0.6*
Ed	S2	3.3±0.6
Ed	Ed	96.7±0.6

Transfected or untransfected S2 cells were DiI-labeled or were left unlabeled and were separately induced with $CuSO_4$. Labeled and unlabeled cell populations were mixed with each other at a ratio of 1:1 (final cell concentration was 1.5×10^6 cells/ml), subsequently aggregated (as described in the Materials and Methods) and analyzed for the presence of labeled cells in cell aggregates. Shown is the average percentage of labeled cell aggregates from three independent experiments. For each combination of labeled and unlabeled cells, 100 cell aggregates of 10 cells or larger were analyzed in each experiment. An aggregate was considered to be 'labeled' if it contained three or more DiI-labeled cells. Cell combinations that are marked with an asterisk contained some aggregates that consisted entirely of DiI-labeled cells (see the insert in Fig. 5C). These aggregates contained only Ed- or Fasciclin 1-expressing cells and are therefore not included in the quantitative evaluation that is presented in this table.

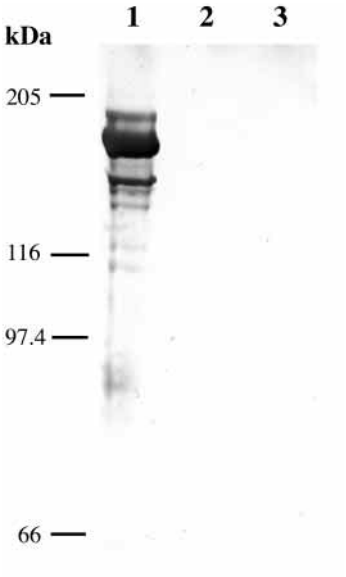

Fig. 6. Co-immunoprecipitation of Ed and Neuroglian protein from mixed S2 cell aggregates. For this co-immunoprecipitation experiments S2 cells expressing Ed were mixed and co-aggregated with either S2 cells expressing Nrg^{GPI} (lanes 1 and 3) or native S2 cells (lane 2). Soluble proteins were immunoprecipitated using the anti-Nrg monoclonal antibody 1B7 (lanes 1 and 2) or a control monoclonal antibody (lane 3). The western blot shown in this figure was analyzed for the presence of Ed protein using the HA.11 monoclonal antibody.

artificial, GPI-anchored form of Nrg was used for these co-immunoprecipitation experiments. S2 cells that expressed Ed or Nrg^{GPI} were mixed, co-aggregated and subsequently extracted with Triton X-100. Soluble proteins and protein-complexes were immunoprecipitated with an anti-Nrg or a control antibody and analyzed on western blots for the presence of Ed protein (Fig. 6). Ed protein was efficiently co-immunoprecipitated together with Nrg^{GPI}, suggesting that a stable and tight interaction is formed between these two adhesive molecules.

Ed acts as receptor in the signal-receiving cells

Thus far, we demonstrated a genetic interaction between *ed* and *nrg* and subsequently their direct heterophilic trans-binding. The synergistic effect of *ed* and *nrg* could be caused by an unidirectional signaling mechanism with either Ed as the receptor (and Nrg as the ligand) or Nrg as the receptor (and Ed as the ligand). Another possibility is that both Ed and Nrg act as receptor molecules (with Nrg and Ed as ligands, respectively) in mediating a bi-directional signaling process. To distinguish between these three possibilities, we used the UAS-Gal4 system to co-express *ed* and nrg^{GPI}, an artificial isoform of Nrg that lacks the intracellular Nrg domain, in the developing *Drosophila* eye disc (Fig. 7A). Overexpression of nrg^{GPI} alone caused no phenotype (Fig. 7C,F,I). However, the synergistic effect between Ed and Nrg on the percentage of ommatidia lacking photoreceptor and cone cell was fully retained for this genetic combination (Fig. 7D,G,J; Table 1). By contrast, co-expression of native nrg^{180} and a truncated artificial isoform of Ed, which lacks the intracellular Ed domain (ed^{intra}, see Fig. 7B), did not exhibit a genetic synergy in the eye disc (data not shown). Similar results were obtained when ed^{intra} and either nrg^{167} or nrg^{GPI} were co-expressed (data not shown). This indicates that the intracellular domain of Ed is essential for repressing EGFR signaling.

In summary, our results suggest that in this context Nrg primarily functions as a heterophilic ligand of Ed and thereby activates Ed in the signal-receiving cell. As a result of its interaction with Ed Nrg would antagonizes EGFR signaling non-autonomously. By contrast, there is no evidence from our experimental assay system for suggesting any signaling from Ed to Nrg. Consistent with this model, we found that the ectopic expression of ed^{C50}, which contains only the transmembrane domain and the last 50 amino acids of the Ed intracellular domain, but lacks the extracellular Ed domain, also causes a reduced number of photoreceptor and cone cells (Fig. 7E,H,K; Table 1).

DISCUSSION

Ed was postulated to engage in homophilic interactions and to initiate a novel, as yet unknown, pathway to antagonize EGFR signaling (Bai et al., 2001). We demonstrate the homophilic adhesive activity of Ed and identify Nrg as a heterophilic binding partner of Ed (Fig. 8). In addition, Nrg and Ed are colocalized in the developing *Drosophila* eye disc. *Nrg* and *ed* act synergistically to inhibit EGFR signaling. This effect requires the intracellular domain of Ed, but not that of Nrg. Taken together, our results support a model whereby Nrg functions as a heterophilic ligand of Ed and activates Ed in the signal-receiving cells to antagonize EGFR signaling.

Nrg is a heterologous ligand of Ed

L1-type CAMs play important roles during neuronal development by mediating axon guidance through homophilic or heterophilic interactions. In vertebrates, a large number of extracellular, heterophilic ligands of L1 family members have been identified (for reviews, see Hortsch, 1996; Hortsch, 2000). Among vertebrate heterophilic ligands of L1-type proteins are several other Ig-domain CAMs, e.g. axonin 1/TAG1, NCAM, F3/F11 and DM-GRASP. The *Drosophila* L1 family member Nrg has a homophilic adhesive activity and recruits ankyrin to the cell contacts by an adhesion-dependent mechanism. We report that Ed is the first identified heterophilic, extracellular partner of Nrg. In this context, Nrg

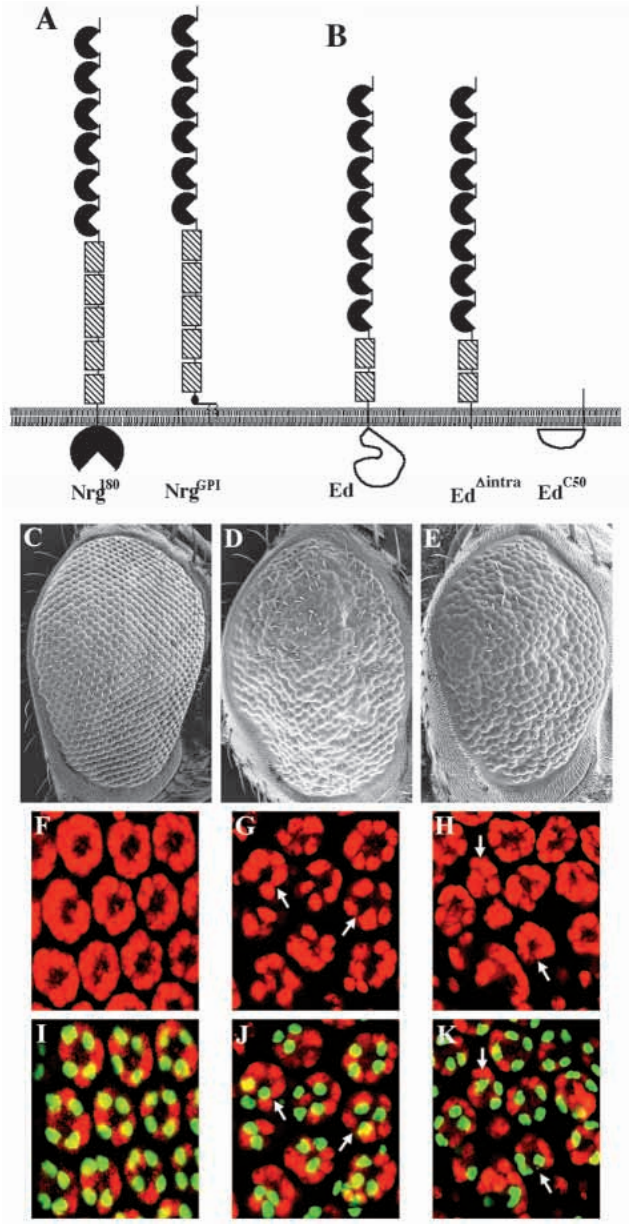

Fig. 7. Ed acts in the signal-receiving cells. (A) Nrg derivatives: Nrg^{180} is the full-length neuron-specific protein isoform and contains the Nrg extracellular, the Nrg transmembrane (TM) and the Nrg^{180} intracellular domain. Nrg^{GPI} contains only the Nrg extracellular domain and is anchored to the plasma membrane by the Fasciclin 1 GPI moiety (Hortsch et al., 1995). (B) Ed derivatives: Ed contains the complete extracellular and TM Ed domains, followed by the entire 315 amino acid intracellular Ed domain. $Ed^{\Delta intra}$ lacks the intracellular Ed domain. Ed^{C50} contains the Ed TM domain and the last C-terminal 50 amino acid of Ed. (C-E) Scanning electron micrographs of adult eyes. (F-K) Eye discs stained for the photoreceptor marker ELAV (red, F-H) and cone cell marker Cut (green, I-K). (C,F,I) *GMR-GAL4/UAS-nrg^{GPI}*; (D,G,J) *GMR-GAL4/UAS-ed/UAS-nrg^{GPI}*; (E,H,K) *GMR-GAL4/UAS-ed^{C50}*. (I-K) Upper focal planes of (F-H), respectively. Overexpression of *nrg^{GPI}* (C,F,I) alone causes no apparent phenotype at 25°C. However, co-expression of both *ed* and *nrg^{GPI}* results in a severe rough eye phenotype (D). A similar phenotype is induced by the overexpression of *ed^{C50}* (arrows in H,K). Arrows in G,J indicate ommatidia contain fewer photoreceptor and cone cells, respectively.

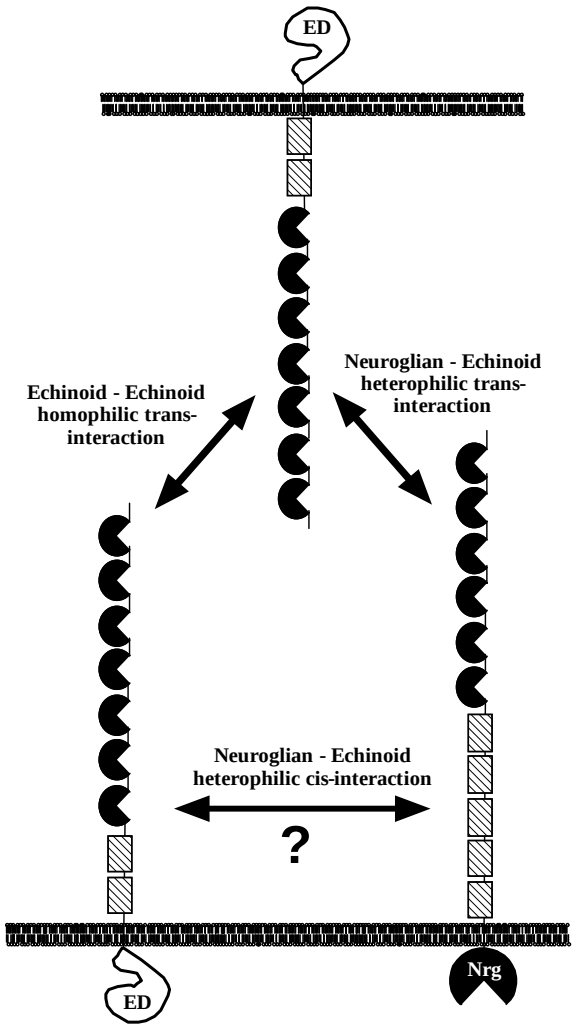

Fig. 8. Model of Echinoid-Neuroglian homophilic and heterophilic interactions. Echinoid and Neuroglian are both homophilic CAMs. They also trans-interact with each other via a heterophilic mechanism. Whether they are also able to cis-interact in the same plasma membrane is currently unknown. Most significantly, L1-type proteins are cell-autonomous, positive regulators of EGFR signaling, whereas Ed negatively influences EGFR function via its cytoplasmic domain. By interacting with Ed that is expressed on neighboring cells Neuroglian can act as an autonomous activator and as a non-autonomous inhibitor of EGFR signaling.

functions as a ligand to activate Ed in the signal-receiving cells. This unidirectional signaling mechanism from Nrg to Ed is further supported by the observation that overexpression of *ed^{C50}* alone can reduce EGFR signaling. By contrast, co-expression of *nrg^{180}* and *ed^{intra}* does not exhibit any genetic synergy in influencing EGFR signaling. Thus, our results fail to support a bi-directional signaling mechanism from Ed to Nrg. Because we do not know whether the intracellular domain of Ed may also be required for signaling out and for activating Nrg in neighboring cells, a signaling process from Ed to Nrg still remains a possibility. The overexpression effect of *ed^{C50}* on the EGFR signaling varies between different lines and tends to be weaker than that observed for *ed* and *nrg* co-expression. It is not clear whether this simply reflects differential

expression levels for Ed^{C50} or whether it lacks the full activity of a wild-type Ed.

The non-neuronal isoform of Nrg (Nrg^{167}) is expressed in the non-neuronal, epithelial cells of eye imaginal discs. It exhibits a similar effect on Ed (and thereby the EGFR signaling pathway) as does the neuronal Nrg isoform (Nrg^{180}), which is expressed by the photoreceptor cells (data not shown). Therefore, Nrg^{167} is probably the major Nrg isoform that inhibits the intrinsic EGFR signaling for basally located, undifferentiated cells. Although our S2 cell mixing experiments clearly show that Ed and Nrg protein interact with each other in a trans-type modus, our results neither prove nor disprove that they might also interact in a cis-type modus. In fact, some Ig-domain CAMs, such as axonin 1/TAG1, interact with L1-type proteins exclusively in a functional cis-type interaction (Malhotra et al., 1998).

Nrg is an autonomous activator of RTK

Genetic evidence indicates that Nrg is a cell-autonomous, positive regulator of EGFR signaling in neuronal cells that express both Nrg and EGFR (Garcia-Alonso et al., 2000) (R.I. and M.H., unpublished). However, in the developing *Drosophila* eye disc Nrg functions non-autonomously as a ligand of Ed and activates Ed in the neighboring cells to repress downstream EGFR signaling. Thus, depending on the cellular context, Nrg can act both as an autonomous activator, as well as a non-autonomous inhibitor of the EGFR signaling pathway.

Autonomous versus non-autonomous effects of *ed* on EGFR signaling

Our previous genetic mosaic analysis indicated that *ed* acts in a cell non-autonomous manner (Bai et al., 2001). As the intracellular domain of Ed is required for EGFR signal repression, we propose that through its homophilic interaction Ed transmits a negative signal in the receiving cell and antagonizes the EGFR pathway. In this study, we demonstrate a homophilic adhesive activity of Ed, and we further show that *ed* also acts autonomously as a heterophilic receptor of Nrg. Thus, Ed appears to influence EGFR signaling through both homophilic (non-autonomous) and heterophilic (autonomous) interactions, but the relative contribution derived from either interaction is unknown. Flies that are mutant for *ed* have extra photoreceptor and cone cells. By contrast, when shifting temperature-sensitive nrg^3 larvae to the restrictive temperature during the third instar larval stage, we did observe wild-type number of Elav- and Cut-positive cells (data not shown). Therefore, the Nrg-mediated heterophilic activity of Ed in repressing EGFR signaling appears to be redundant with the homophilic activity of Ed.

Further studies are required to reveal the molecular mechanism by which *ed* inhibits the EGFR signaling pathway. Equally, with *ed* and nrg widely expressed in the developing *Drosophila* eye disc, it remains to be revealed how the two opposing effects of *nrg* on EGFR activity might contribute to a differential cellular segregation and the development of different ommatidial cell types.

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