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 LICAM gene result in mental retardation and other neurological phenotypes, for which summarily the term CRASH syndrome has been coined. This emphasizes the importance of LICAM 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 Nrg180 isoform is neuron specific, whereas the Nrg167 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 nrg1 (Hall and Bieber, 1997), UAS-ed and UAS-edint (Bai et al., 2001). The UAS-nrg180, UAS-nrg167 and the UAS-nrgGP1 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-edC50 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 Nrg180 and NrgGP1 have been described previously (Hortsch et al., 1995). The pRmHa3-Drosophila Fasciclin 1 plasmid was constructed by ligating a 3.0 kb EcoRI Fasciclin 1 cDNA fragment into the EcoRI restriction site of the pRmHa3 vector. The Echinoid cDNA was subcloned into pRmHa3 as a 4.3 kb SmaI/Sall fragment and subsequently modified by the removal of a 3’ 0.5 kb SstI/Sall 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 pCoHygro 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 CuSO4 and analyzed by Western blotting for high expression of the transfected cDNAs. Selected lines, designated S2:Ed, S2:Nrg180, S2:NrgGP1 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 CuSO4. Labeled, induced cells were mixed with unlabeled, induced cells as indicated to a final concentration of 1.5×10^5 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 I87, 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 CuSO₄ and mixed and incubated on a shaking platform as indicated. A total of $10^6$ cells were collected by centrifugation for each immunoprecipitation and solubilized in a buffer containing 1.25% Triton 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 ommatidium 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 nrg180 or the non-neuronal nrg167 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 nrg180 (or nrg167) 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 Fasciculin 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. nrg1 is a nrg null allele (Hall and Bieber, 1997). A reduction in half of the

![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-nrg180; (D,H,L) GMR-GAL4/UAS-ed+UAS-nrg180. (I-L) The upper focal planes of E-H, respectively. There are seven photoreceptor (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), ommatidia 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 nrg180 (C,G,K) alone causes no phenotype at 25°C. However, co-expression of both ed and nrg180 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 and cone cells (arrows in L).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Photoreceptor loss (%)</th>
<th>Cone cell loss (%)</th>
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<tr>
<td>GMR&gt;ed</td>
<td>16.2 (n=198)</td>
<td>12.1 (n=993)</td>
</tr>
<tr>
<td>GMR&gt;Nrg180</td>
<td>0.0 (n=130)</td>
<td>0.0 (n=150)</td>
</tr>
<tr>
<td>GMR&gt;Nrg167</td>
<td>0.0 (n=108)</td>
<td>0.0 (n=145)</td>
</tr>
<tr>
<td>GMR&gt;ed + Nrg180</td>
<td>50.7 (n=223)</td>
<td>69.0 (n=423)</td>
</tr>
<tr>
<td>GMR&gt;ed + Nrg167</td>
<td>83.8 (n=370)</td>
<td>71.9 (n=178)</td>
</tr>
<tr>
<td>GMR&gt;ed+GPI</td>
<td>31.1 (n=160)</td>
<td>26.3 (n=137)</td>
</tr>
<tr>
<td>nrg1+/+; GMR&gt;ed</td>
<td>15.8 (n=476)</td>
<td>9.5 (n=1550)</td>
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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 ($P<0.0001$; $\chi^2$ test).
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. 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 2.5×10⁵ 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.
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
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

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

<table>
<thead>
<tr>
<th>Unlabeled cells</th>
<th>Labeled cells</th>
<th>% of cell aggregates with DiI-labeled cells</th>
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<tbody>
<tr>
<td>Nrg180</td>
<td>S2</td>
<td>3.3±0.6</td>
</tr>
<tr>
<td>Nrg180</td>
<td>Ed</td>
<td>97.0±0.8</td>
</tr>
<tr>
<td>Nrg180</td>
<td>Fas1</td>
<td>4.0±1.0*</td>
</tr>
<tr>
<td>Fas1</td>
<td>S2</td>
<td>2.7±0.6</td>
</tr>
<tr>
<td>Fas1</td>
<td>Ed</td>
<td>3.3±0.6*</td>
</tr>
<tr>
<td>Ed</td>
<td>S2</td>
<td>3.3±0.6</td>
</tr>
<tr>
<td>Ed</td>
<td>Ed</td>
<td>96.7±0.6</td>
</tr>
</tbody>
</table>

Artificial, GPI-anchored form of Nrg was used for these co-immunoprecipitation experiments. S2 cells that expressed Ed or NrgGPI 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 NrgGPI, 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 nrgGPI, an artificial isoform of Nrg that lacks the intracellular Nrg domain, in the developing Drosophila eye disc (Fig. 7A). Overexpression of nrgGPI alone caused no phenotype (Fig. 7C,F,1). 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; Table 1). By contrast, co-expression of native nrg180 and a truncated artificial isoform of Ed, which lacks the intracellular Ed domain (ed intr, see Fig. 7B), did not exhibit a genetic synergy in the eye disc (data not shown). Similar results were obtained when ed intr and either nrg167 or nrgGPI 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 edC50, 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).
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 edC50 alone can reduce EGFR signaling. By contrast, co-expression of nrg180 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<sup>C50</sup> or whether it lacks the full activity of a wild-type Ed.

The non-neuronal isoform of Nrg (Nrg<sup>167</sup>) 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<sup>180</sup>), which is expressed by the photoreceptor cells (data not shown). Therefore, Nrg<sup>167</sup> 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 (García-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 (R.I. and M.H., unpublished). Therefore, Nrg167 is probably the major Nrg isoform that is expressed by the photoreceptor cells (data not shown).

**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<sup>3</sup> larvae to the restrictive temperature, 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).

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