INTRODUCTION

FGF and EGF are extracellular signaling factors that control various aspects of morphogenesis, patterning and cellular proliferation in both invertebrates and vertebrates. These ligands act through high-affinity transmembrane receptors with an intracellular tyrosine kinase moiety. In Drosophila, a single EGF receptor homolog, encoded by the Egfr gene, and two FGF receptor homologs, encoded by breathless and heartless, have been identified (Beiman et al., 1996; Gisselbrecht et al., 1996; Glazer and Shilo, 1991; Klämbt et al., 1992; Livneh et al., 1985). Upon reception of the extracellular signals, these receptor tyrosine kinases (RTKs) activate the ras/MAPK signaling pathway. Many of the molecular components of this signaling pathway, such as ras GTPase, raf kinase and MAPK, are shared among different RTKs. Thus information from multiple extracellular signals are interpreted using the same molecular cassette (reviewed by Schlessinger, 1993).

While the intracellular tyrosine kinase domain is conserved, the extracellular domains of the RTKs are specialized for their diverse inputs. Of these three receptors, the EGF receptor is the most complex, as it is responsive to three tissue-specific activating ligands, Spitz, Gurken and Vein (reviewed by Perrimon and Perkins, 1997; Schweitzer and Shilo, 1997). Spitz acts in the ventral ectoderm, the midline of the central nervous system (CNS), the chordotonal organs and imaginal discs. Gurken is the key determinant in patterning the ovarian follicle cells. The third ligand, Vein, functions during wing vein formation and in the attachment of the embryonic muscles. Unlike Spitz and Gurken, which are members of the TGFα family, Vein shows similarity to neuregulins (Neuman-Silberberg and Schüpbach, 1993; Rutledge et al., 1992; Schnepf et al., 1996). The only known ligand for the FGF receptors is the Branchless FGF, which acts through Breathless during tracheal branching (Sutherland et al., 1996). In most of these systems, the distribution of the activating ligands, which is governed mainly by their synthesis, processing and diffusion, is the primary factor controlling the differential behavior of the responding cells. In addition, the EGF signaling pathway is subject to negative feedback regulation. The production of the EGF-like antagonist Argos is dependent on EGFR signaling (Schweitzer et al., 1995; Golembo et al., 1996).
and a hyperactivation of EGFR results in a downregulation of receptor expression (Sturtevant et al., 1994). Elucidating the mechanisms that regulate RTK signaling is key to the understanding of how extracellular signals achieve precise cellular responses.

Recently, we have reported that Sprouty (SPRY) acts as a novel extracellular antagonist of FGFR signaling during tracheal development (Hacohen et al., 1998). Similar to Argos, which is induced by EGFR signaling (Golembo et al., 1996), spry expression is induced by the FGFR pathway that it inhibits. Here we show that spry is also expressed in other developmental systems such as the eye imaginal disc, the embryonic chordotonal organ precursors and the midline glia. In all of these systems, EGFR receptor signaling is known to participate in the control of the correct number of neurons or glia. We examine spry function in several of these tissues, focusing on its regulation of EGF-induced neuronal differentiation in the eye.

The Drosophila compound eye is a stereotyped array of 800 unit eyes or ommatidia, each of which comprises an invariant number of cells; 8 photoreceptor neurons (named R1 through R8) and 12 non-neuronal accessory cells. The photoreceptor cells are recruited by a stereotyped sequence of inductive interactions, mediated by two RTKs, EGFR and Sevenless (SEV). EGFR signaling is required for the recruitment or maintenance of all ommatidial cells (Freeman, 1996; Kumar et al., 1998; Xu and Rubin, 1993), whereas SEV is required specifically for the neuronal specification of the R7 cell (Tomlinson and Ready, 1986). Activation of EGFR by its ligand Spitz (Freeman, 1994; Tio et al., 1994) or SEV by BOSS (Van Vactor et al., 1991) activates the ras/MAPK pathway, which ultimately impinges on a number of nuclear targets such as the Pointed Ets-domain transcription factor (Brunner et al., 1994; O’Neill et al., 1994) and results in the initiation of neuronal development (for review see Dickson, 1995; Zipursky and Rubin, 1994).

In addition to the presumptive photoreceptor neurons, two cell types within an ommatidium can be induced to become neurons upon ectopic activation of the RTK signaling pathways: the lens-secreting non-neuronal cone cells and the mystery cells, two cells transiently associated with the ommatidial precluster (Tomlinson and Ready, 1987; Wolff and Ready, 1991a). Ectopic activation of the ras/MAPK pathway in these cells is sufficient to elicit their differentiation as neurons (Basler et al., 1991; Dickson et al., 1992; Fortini et al., 1992). In normal development, ectopic activation of the pathway is prevented by the action of several negative regulators of RTK signaling. Gap1 encodes a GTPase activating protein that is thought to act by decreasing the levels of activated Ras1 in the eye, whereas yan encodes for an Ets-domain protein that interferes with Pointed signaling (Gaul et al., 1992; Lai and Rubin, 1992). Mutations in either of these loci causes the cone cells and mystery cells to differentiate as photoreceptor neurons. Absence of a third factor, Argos, a secreted antagonist of EGFR signaling, results in a transformation of mystery cells into outer photoreceptor cells and in the recruitment of extra cone and pigment cells, but does not affect the development of the presumptive cone cells (Freeman et al., 1992b; Kretzschmar et al., 1992; Okano et al., 1992).

Here we show that mutation in spry causes a transformation of non-neuronal cone cells into R7 cells and of mystery cells into outer photoreceptor cells, a phenotype identical to the one seen with hyperactivation of EGFR signaling. SPRY can also antagonize EGFR signaling in other tissues such as the midline glia, the chordotonal organs, the wing and the ovarian follicle cells. Since SPRY acts as an antagonist of FGFR-mediated signaling during tracheal development, the EGFR and FGFR signaling pathways appear to share an unexpected degree of conservation in the extracellular compartment.

MATERIALS AND METHODS

Genetics

We identified five EMS-induced alleles of spry in a genetic screen for dominant suppressors of the ro-svp eye phenotype (Kramer et al., 1995). Misexpression of svp in R2/R5 by the ro-svp transgene causes mis specification of these cells, resulting in compromised neuronal induction of R1/R6. spry dominantly suppresses this phenotype by rescuing the neuronal differentiation of R1/R6, without restoring the mis specification of R2/R5 (data not shown). The screen was carried out essentially as described previously for suppressors of the sev-svp phenotype (Kramer et al., 1995), except that mutagenized st e males were crossed to CyO, 2 × P[ro-svp]/Sp virginal females. 50,000 F1 progeny were screened and sold (ro-svp) on the third chromosome were balanced over TM3, ry, 2 × P[ro-svp]. Mutations on the third chromosome were mapped meiotically using the rucuca chromosome (ru, h, th, st, cu, sr, e, ca). Deficiency mapping of spry was carried out with Df(3L)HR119, Df(3L)HR232, Df(3L)HR370 (Wohhwill and Bonner, 1991), Df(3L)A466 (Kulkarni et al., 1994) and Df(3L)1226 (gift of S. Paine-Saunders and J. Frisstrom). Df(3L)HR119 (63C1; 63D3), Df(3L)HR232 (63C6; 63E) and Df(3L)1226 (63C6; 63E1) failed to complement the lethality of spry EMS alleles, whereas Df(3L)HR370 (63A1; 63D1) and Df(3L)A466 (63D1-2; 64B1-2) complemented it. The eye phenotypes of various spry alleles in trans to each other were indistinguishable from those over a deficiency of the locus, suggesting that all spry alleles tested were amorph or strong hypomorphs (Table 1).

spry254 clones and clones double mutant for spry254 and a strong hypomorphic allele of sina (sina2; Carthew and Rubin, 1990) or a null allele of argos (argos287; Okabe et al., 1996) were generated using the FRT technique (Xu and Rubin, 1993). spryAS clones in a sevc background were generated in animals of the genotype w1118, sevF2, P[hs-FLP]; spryAS, P[FRT]80B/P[w1]70C, P[FRT]80B. Control animals were raised without heat shock. Gap1AR and yam2 are described in Gao et al. (1992), and Lai and Rubin (1992), respectively. pointed8B is an amorph (Klämbt, 1993) and Ras1C26/1C31 is a combination of Ras1 hypomorphic alleles that survives to the third instar larval stage (our unpublished observation). breathless10, heartless89 and branchless84 are amorphs or strong hypomorphs (Klämbt et al., 1992; Shishido et al., 1997; Sutherland et al., 1996). For ectopic expression studies, UAS-spry was placed under the control of elav-GAL4 C155 (Lin and Goodman, 1994), sev-GAL4 K25 (Brunner et al., 1994), en-GAL4 (gift of A. Brand and N. Perrimon), CY2 (Queenan et al., 1997) and BH1 (Schüpbach and Wieschaus, 1998). sev-Ras1N17, UAS-spry and GMR-argos lines are described in Allard et al. (1996), Hacohen et al. (1998) and Sawamoto et al. (1998), respectively.

Histology and histochemistry

Fixation and sectioning of adult heads and antibody staining of imaginal discs were performed as described (Tomlinson and Ready, 1987), except that, in some instances, the peripodial membrane was not removed from imaginal discs. SPRY protein was detected using two polyclonal rabbit antisera; 26A was raised against full-length...
**Sprouty antagonizes FGF and EGF signaling**

**Table 1. Comparison of recessive phenotypes of different spry allelic combinations**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Average no. of outer PRCs</th>
<th>Average no. of R7 cells</th>
<th>Ommatidia scored</th>
</tr>
</thead>
<tbody>
<tr>
<td>spry^A5/spry^A5</td>
<td>6.09</td>
<td>3.09</td>
<td>120</td>
</tr>
<tr>
<td>spry^A5/Df(3L)1226</td>
<td>6.09</td>
<td>2.71</td>
<td>82</td>
</tr>
<tr>
<td>spry^226Df(3L)1226</td>
<td>6.17</td>
<td>3.26</td>
<td>115</td>
</tr>
<tr>
<td>spry^254spry^226</td>
<td>5.96</td>
<td>3.04</td>
<td>190</td>
</tr>
<tr>
<td>spry^F7spry^226</td>
<td>6.04</td>
<td>3.23</td>
<td>114</td>
</tr>
<tr>
<td>spry^254 clone</td>
<td>6.14</td>
<td>3.08</td>
<td>126</td>
</tr>
<tr>
<td>sev^Δ2, spry^234 clone</td>
<td>6.15</td>
<td>2.96</td>
<td>96</td>
</tr>
</tbody>
</table>

Retinular phenotypes of animals of the indicated genotypes were examined in apical tangential sections and average numbers of outer PRCs and R7 cells per ommatidium calculated for each genotype. For the wild type, the average number of outer PRCs would be 6.0 and the average number of R7 cells would be 1.0.

SPRY protein and 32C against a peptide extending from amino acid 63 through 462 (Hacohen et al., 1998). An affinity-purified antibody against BarH1/BarH2 proteins (Higashijima et al., 1992), a monoclonal antibody against ELAV protein and monoclonal antibody 22C10 (Fujita et al., 1982) were gifts of the Saigo, Rubin and Goodman laboratories, respectively. Monoclonal antibodies against β-galactosidase were purchased from Promega. The expression pattern of enhancer trap insertion 9143 was detected using anti-β-galactosidase mAb 40-1a (obtained from Developmental Studies Hybridoma Bank) and the Vectastain elite kit (Vector laboratories). Embryo stainings were carried out according to Patel (1994).

The following lacZ enhancer trap lines were used as cell-type-specific markers: X81 and AE127 are enhancer trap insertion in the rhomboid gene (Freeman et al., 1992a) and thesvp gene (Mlodzik et al., 1990), respectively. P82 expresses lacZ in R3, R4 and R7 (Kramer et al., 1995), H214 is an enhancer trap insertion that expresses β-galactosidase at high levels only in the R7 cell (Mlodzik et al., 1992) and AA142 is an enhancer trap insertion with expression in the midline glia (Klämbt et al., 1991).

In situ hybridizations to eye imaginal discs and embryos were performed essentially as described by O’Neill and Bier (1994), and Lehmann and Tautz (1994). RNA probes were transcribed from a 1 kb and a 2.2 kb EcoRI fragment of the longest spry cDNA cloned into BSSK* (Hacohen et al., 1998) using the Boehringer Mannheim kit according to the instructions of the manufacturer.

**RESULTS**

**spry is required to prevent neuronal induction of non-neuronal cells in the retina**

Animals homozygous for any of the EMS-induced alleles of spry or spry^A5 died as pharate adults. The rareescapers had eyes that were similar in size to wild-type eyes but had a disorganized exterior. A majority of the ommatidia in these animals contained supernumerary photoreceptor neurons, which by their morphology were R7 cells. In addition, some of the extra photoreceptors resembled outer photoreceptor neurons (Fig. 1C; Table 1). Examination of the early stages of neuronal development in the eye imaginal disc with molecular markers revealed that the supernumerary photoreceptors originated from non-neuronal cone cells and mystery cells that had assumed R7 and R3/R4 fates, respectively (Fig. 2). Neuronal markers were inappropriately activated in cone and mystery cells at the same time in development as in the normal photoreceptors, implying that the defect in the mutant occurs at the normal time of photoreceptor induction. Thus, spry functions in the eye imaginal disc to prevent neuronal induction of these non-neuronal cells.

**spry functions as a dosage-dependent inhibitor of neuronal induction.** In the heterozygous condition, all spry alleles examined contained 1-7% of ommatidia with an extra R7 cell or an occasional gain or loss of an outer photoreceptor cell (Fig. 1B; Table 2). Conversely, increased levels of SPRY in the developing eye disc inhibited the induction of normal photoreceptor cells. Animals that expressed UAS-spry under either the sev-GAL4 or the elav-GAL4 driver had small disorganized eyes. The majority of ommatidia lacked one or more outer photoreceptor cells of the R3/R4/R1/R6 subtype (Fig. 3A-C). The R7 cell was missing in 11±4% of sev-GAL4/UAS-spry, and in 18±6% of elav-GAL4/UAS-spry ommatidia. Development of the R2/R5 and R8 photoreceptors were disrupted in these animals.

**Table 2. Dominant interactions between spry and the intracellular negative regulators of ras signaling**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Ommatidia with extra R7 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>spry^254+</td>
<td>6.3±2.2</td>
</tr>
<tr>
<td>yan^1/p+</td>
<td>0.4±0.4</td>
</tr>
<tr>
<td>Gap1^2/p+</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>yan^1/p+; spry^226/p+</td>
<td>61±21</td>
</tr>
<tr>
<td>Gap1^2/p+; spry^226/p+</td>
<td>43±3</td>
</tr>
</tbody>
</table>

**Fig. 1.** The eye phenotype of spry loss-of-function alleles. (A) A tangential section of a wild-type eye at the apical level shows a regular array of ommatidia. Each ommatidium contains eight photoreceptor neurons (R1-8), which can be identified by the size and position of their darkly stained light-gathering structure (rhodopsomes). Based on their morphology, the photoreceptor cells (PRCs) can be subdivided into three classes. The outer PRCs (R1 through R6) have large rhodopsomes that extend through the entire depth of the retina and are arranged in a trapezoidal array. R7 and R8 have small rhodopsomes that project into the center of the ommatidium. In this apical section, only the rhodopsome of the R7 cell is visible. R8 is underlying the R7 cell in a more basal position. (B) spry loss-of-function alleles had a weak dominant phenotype, shown here is an apical tangential section of a spry^226+ eye. In eyes of animals heterozygous for a spry EMS allele, on average 3.4% of ommatidia had an extra R7 cell (arrowhead) with some variation between different alleles (spry^226: 7.2%, spry^G5: 5.5%, spry^F7: 1.7%, spry^254: 1.2%, spry^G1: 1.0%). spry^A5 showed the weakest dominant phenotype: 0.8% of ommatidia had an extra outer PRC. (C) An apical tangential section of an eye of an animal homozygous for spry^A5, a putative amorph of spry. Most ommatidia contained between one and four extra R7-like photoreceptor cells, and 27% of ommatidia had one or two extra outer photoreceptor cells. In addition, a small percentage of ommatidia had less than the normal number of photoreceptor cells.
spry antagonizes neuronal induction by the EGFR pathway

Two RTK signaling pathways are required for neuronal induction of photoreceptor cells, the EGFR pathway and the SEV pathway. While SEV signaling is required only for R7 development, EGFR signaling is necessary for the specification and maintenance of all photoreceptor neurons. Overexpression of spry results in a loss of mainly outer photoreceptor cells, which is also seen with compromised EGFR signaling (Freeman, 1996; Kumar et al., 1998). Conversely, expression of an activated form of EGFR (Queenan et al., 1997) causes excess neuronal differentiation, which is reminiscent of the spry loss-of-function phenotype (data not shown). These results are consistent with the idea that spry antagonizes EGFR signaling.

Since hyperactivation of SEV signaling also produces excess
Fig. 4. Epistatic relationship of *spry* with components of the ras signaling pathway. (A,B) Apical tangential sections of a *sev*Δ; *spry*ΔΔ clone in a *sev*ΔΔ background (A) and of a *spry*ΔΔ, *sina*Δ double mutant clone (B). Clones were marked by the absence of a functional white gene. (A) Extra R7 cells in *spry*ΔΔ mutant ommatidia are independent of *sev* gene function. (B) In contrast, extra R7 cells in *spry*ΔΔ mutant ommatidia require *sina* function. Out of 97 *spry*ΔΔ, *sina*Δ ommatidia scored, none contained an R7 cell. The small central rhabdomeres visible in B were shown to be apically displaced R8 cells by serial sectioning. (C,D) Apical tangential eye sections of *sevE-Ras1*N17/+ (C) and *sevE-Ras1*N17/+; *spry*ΔΔ/*spry*ΔΔ eyes (D). In the eyes of animals expressing one copy of *sevE-Ras1*N17, a dominant negative ras, the R7 cell is lost in approximately half of the ommatidia (C). *sevE-Ras1*N17 effectively suppresses the appearance of extra R7 cells in *sevE-Ras1*N17/+; *spry*Δ/+/*spry*ΔΔ eyes; the average number of R7 cells per ommatidium was decreased to 0.95±0.04 (D, compare Fig. 1C for *spry*ΔΔ phenotype). (E-L) *spry* suppresses the effect of overexpression of Argos. (E-H) Scanning electron micrographs of adult retinae. (I-L) ELAV expression in the pupal eye. In animals carrying two copies of the GMR-argos transgene, photoreceptor neurons undergo apoptosis, resulting in small eyes with fewer photoreceptor neurons per ommatidium (F,I), compared to the wild type (E,I). The eyes of animals homozygous for *spry*ΔΔ are normal in size, but have additional photoreceptor neurons (G,K). The eye phenotype of GMR-argos; *spry*ΔΔ animals is similar to the one of *spry*ΔΔ animals; the eye size is normal and extra photoreceptor neurons are present in most ommatidia (H,L). These animals carry two copies of the GMR-argos transgene and are homozygous for *spry*ΔΔ.

Photoreceptor neurons, we tested whether *spry* acted by antagonizing the SEV RTK. If the extra R7 photoreceptors in *spry* mutants resulted from overactivity of SEV, then the affected cells should require *sev* for their development. However, we found that removing *sev* had little or no effect on R7 development in the *spry* mutant background: the average number of R7 cells was 3.08 for *spry*ΔΔ/ΔΔ eyes; the average number of R7 cells per ommatidium was decreased to 0.95±0.04 (D, compare Fig. 1C for *spry*ΔΔ phenotype). (E-L) *spry* suppresses the effect of overexpression of Argos. (E-H) Scanning electron micrographs of adult retinae. (I-L) ELAV expression in the pupal eye. In animals carrying two copies of the GMR-argos transgene, photoreceptor neurons undergo apoptosis, resulting in small eyes with fewer photoreceptor neurons per ommatidium (F,I), compared to the wild type (E,I). The eyes of animals homozygous for *spry*ΔΔ are normal in size, but have additional photoreceptor neurons (G,K). The eye phenotype of GMR-argos; *spry*ΔΔ animals is similar to the one of *spry*ΔΔ animals; the eye size is normal and extra photoreceptor neurons are present in most ommatidia (H,L). These animals carry two copies of the GMR-argos transgene and are homozygous for *spry*ΔΔ.

**Spry is expressed near EGF signaling centers during neuronal induction**

The expression pattern of *spry* in developing eye imaginal discs during the time of the neuronal inductions was determined by RNA in situ hybridization. Induction of the eight photoreceptor cells in each ommatidium occurs in the 5 or 6 rows posterior to the morphogenetic furrow in the third instar larval eye imaginal disc. *spry* mRNA was found in a stripe of cells corresponding to rows 1 through 5 posterior to the furrow. In addition, there was a second stripe of *spry*-expressing cells in more mature ommatidial preclusters corresponding to rows 8 through 11 (Fig. 5A). The low resolution of in situ hybridization experiments precluded identification of the *spry*-expressing cells in these regions. However, a *spry* lacZ enhancer trap line mimics expression of the gene in other tissues (Hacohen et al., 1998) in addition to the eye imaginal disc, and expression of the reporter can be resolved at cellular resolution. The *spry* reporter was expressed in all photoreceptor neurons, with particularly high levels in photoreceptors R2, R5 and R7. These three cells directly contact the R8 cell, which is the first photoreceptor specified and, along with R2 and R5, presumably constitutes the EGF signaling center. These three cells are the initial source of Spitz (Freeman, 1994; Tio et al., 1994) and they specifically express the *rhomboid* and *Star* genes required to generate the active EGF ligand (reviewed in Freeman, 1997). Lower levels of expression of the *spry* reporter were detected in photoreceptor cells R3/R4/R1/R6 which are located slightly further from the R8 cell (Fig. 5G,H,J), and no expression was detected in the mystery or cone cells located just beyond them. At slightly later stages in ommatidial development, corresponding to rows 8 and beyond behind the furrow, *spry* expression was detected at low levels in cone cells, and it was present at higher levels in cone cells at the pupal stage (Fig. 5J,K). The *spry* reporter was not expressed in *Ras1* mutant eye discs, or in clones mutant for *pointed* (Fig. 5L,M). Thus, as in the embryonic trachea (Hacohen et al., 1998), *spry* is expressed near an RTK.
spry mutations cause phenotypes in the embryonic central and peripheral nervous system similar to those seen with increased EGFR signaling

In addition to the eye imaginal disc, spry is expressed in several other developing tissues known to require EGFR for cell fate induction or tissue patterning. For example, spry is expressed in the anlage of the embryonic chordotonal organs, neural sensory structures that require EGFR signaling for their proper development (Lage et al., 1997; Okabe and Okano, 1997). spry RNA is expressed in cells that become chordotonal organ precursors (COPs), as well as cells that surround the COPs (Fig. 6C). In spry mutants, between 26% and 37% of hemisegments contained an extra chordotonal organ in the lateral cluster (Fig. 6D,E). This is the same phenotype seen in mutants for negative regulators of EGFR signaling, such as argos, Gap1 and yan (Okabe and Okano, 1997; Okabe et al., 1996). Overexpression of spry resulted in the reduction in the number of chordotonal organs (Fig. 6F), similar to the effect of reduced EGF signaling. In contrast, mutations in all of the known FGF ligands and receptors (breathless, heartless, branchless) all had a normal number of chordotonal organs (data not shown). Thus it is unlikely that the spry phenotype is due to hyperactivation of FGF signaling in developing chordotonal organs.

spry is also expressed in several developing midline glial cells in each segment of the embryonic CNS (Hacohen et al., 1998). Both FGF and EGFR pathways are required for development of the midline glia cells, the former for proper cell migration and the latter for cell survival (Dong and Jacobs, 1997; Klämbt et al., 1992; Scholz et al., 1997). We found that spry embryos have extra midline glial cells (Fig. 6A,B), as do embryos in which EGFR signaling is hyperactivated by mutation in the argos gene (Scholz et al., 1997). These results suggest that spry antagonizes EGFR signaling in the developing midline glia and the chordotonal organs as well as in the eye.

Overexpression of spry in the developing wing and ovary mimics Egfr mutant phenotypes

We investigated whether SPRY can antagonize EGFR
Fig. 6. spry phenotype in the midline glia and the chordotonal organ. (A,B) Midline glia of wild-type (A) and spryΔ5 embryos (B), visualized using enhancer trap line AA142. In segments T1 to A7, wild-type embryos had 2.98±0.56 midline glia per segment (n=49), whereas spry mutant embryos had 4.74±0.74 (n=35). (C) Expression of SPRY mRNA in the region that forms chordotonal organ precursors (COPs). The position of five COPs that have already delaminated are indicated by asterisks. These cells express SPRY, but are out of the plane of focus. SPRY mRNA is present in a group of cells that surround these five COPs. Three additional COPs will delaminate from this group of cells. SPRY mRNA can be detected in the tracheal pit (T) at stage 11 and is expressed in the region of primary branches at stage 12 on, as previously described (Hacokhen et al., 1998). (D-F) The effect of spry loss- and gain-of-function on chordotonal organ development. Embryos were stained with mAb 22C10, which recognizes all peripheral neurons. In wild-type embryos, the lateral cluster contains five chordotonal organs with one neuron each (D). In spryΔ5 embryos 26% of hemisegments (n=68) contain an extra chordotonal organ in the lateral cluster (E). In spryΔ24 embryos, this phenotype was seen in 37% of hemisegments. Overexpression of spry in en-GAL4/UAS-spry embryos causes a reduction of the number of chordotonal organs per hemisegment (F). The positions of the neurons of the lateral chordotonal organ are indicated by arrowheads.

Fig. 7. spry overexpression in the wing and in follicle cells. (A) spry enhancer trap line 9143 expression in the wing imaginal disc. spry is expressed in the presumptive notum, proximal wing and the central region of the wing blade (arrowhead). A similar expression pattern was seen with SPRY mRNA (data not shown). (B-E) The effect of spry loss- and gain-of-function on wing vein patterning. (B) Wild-type wing. (C) spry mutant wings often have extra wing vein material (arrowhead), especially in the distal region of L2 (inset). Animals overexpressing spry (BH1/UAS-spry) lack most wing veins (D). In addition the size of the wing is decreased. en-GAL4/UAS-spry animals lack veins in the posterior compartment (E). (F) spry enhancer trap expression in the ovarian follicle cells. spry is expressed first in the posterior follicle cells, then in the dorsal-anterior follicle cells (arrowheads). (G,H) The effect of spry misexpression in the follicle cells. In wild-type embryos, two dorsal appendages arise from the dorsolateral follicle cells (G). Overexpression of spry in all follicle cells using GAL4-line CY2 causes a ventralization of the egg shell, resulting in eggs with a fused thin dorsal appendage (H). The embryos developing from these eggs are likewise weakly ventralized.

signaling in two other tissues where EGFR signaling is known to operate, the developing wing and ovary. In the wing imaginal disc, spry enhancer trap line 9143 is expressed in the presumptive notum, in clusters of cells that give rise to sensory organs in the proximal wing and in the central region of the wing blade (Fig. 7A). spry− mutant wings showed minor vein defects, with occasional extra vein material (Fig. 7C). When spry was overexpressed in the wing imaginal disc, wing veins failed to form in the region of spry overexpression (Fig. 7D,E). In addition, there was a reduction in the size of the wing blade, indicating a reduction in the rate of cell proliferation. These phenotypes are similar to the ones seen with loss of EGFR function (Clifford and Schüpbach, 1989) or in the absence of the EGFR ligand Vein (Simcox et al., 1996).

In the developing ovary, the spry enhancer trap line is expressed first in the posterior-most follicle cells, and then in the dorsal anterior follicle cells overlying the oocyte nucleus (Fig. 7F). These are precisely the regions where EGFR is activated by its ligand Gurken (Gonzalez-Reyes et al., 1995; Roth et al., 1995), suggesting that spry expression is induced by EGFR signaling. When spry was ectopically expressed in all follicle cells, the resultant eggs had a ventralized egg shell and the embryos that developed from the eggs were likewise ventralized (Fig. 7G,H; data not shown). The spry overexpression phenotype mimics the loss of EGFR function in the ovary (Schüpbach, 1987), indicating that SPRY antagonizes EGFR signaling in the follicle cells.
Fig. 8. Genetic mosaic analysis of spry in the eye imaginal disc. (A) Section of a mosaic retina containing a spry254 clone, marked with the white mutation. Note that the spry- mutant phenotype is not rescued at the boundary of the mosaic clone. (B) Results of a mosaic analysis on spry254 mutant clones. The genotypes of individual PRCs were identified by the presence (w+, spry+) or absence (w-, spry-) of pigment granules visible as black dots at the base of rhabdomeres. A total number of 79 mosaic ommatidia of wild-type construction were scored. The table shows the frequencies of w-, spry- PRCs in ommatidia mosaic for spry and w (first column, spry-), and of w-, spry+ PRCs in control clones mosaic for white (second column, w-). The genotypes of the 79 mosaic ommatidia are depicted graphically. Photoreceptor cells R1, R6 and R7 showed an increased requirement for spry function in mosaic ommatidia of wild-type construction. Note however that, in two ommatidia, R1, R6, and R7 were spry- and, in an additional five ommatidia, two of these three cells were spry-.

(C,D) Section (C) and tracing (D) of a sevΔ2, spryΔ5 mosaic clone induced in a sevΔ2 eye. Ommatidia with one or more R7 cells and the normal number of outer PRCs were scored. w+, spry+ PRCs are outlined in white and w-, spry- PRCs in black. Of 140 R7 cells examined, 137 (97.9%) were w-, spry- and 3 (2.1%) were w+, spry+. Of 99 ommatidia scored, 3 (3.0%) had a spry+ R7 cell. In the eyes of control animals of the same genotype raised without heat shock, 0.2% of ommatidia had an extra R7 cell. The frequency of spry+ R7 cells seen in the mosaic analysis and the frequency of R7 cells seen in the control were distinct with P<0.01 (Students t-test). 16 ommatidia were wild-type in appearance, i.e. one R7 cell projected into the center of the ommatidium in the correct position relative to the R8 cell. The frequency of spry+ PRCs in these ommatidia (R1, 56%; R2, 87%; R3, 75%; R4, 75%; R5, 75%; R6, 50%; R7, 0%; R8, 87%) was comparable to the frequency of spry+ PRCs (R1-R6, 66%; R8, 71%) in ommatidia with multiple R7 cells.

SPRY acts in parallel to Argos in antagonizing the EGFR pathway

Many of the processes that are controlled by EGFR signaling are antagonized by the extracellular factor Argos (reviewed in Schweitzer and Shilo, 1997). We therefore tested the genetic relationship between spry and argos. Overexpression of Argos using a GMR-argos transgene causes apoptosis of photoreceptor neurons and a reduced eye structure (Sawamoto et al., 1998). By contrast, GMR-argos; spry- animals have normal-sized eyes that contain excess photoreceptor neurons, similar to spry mutants (Fig. 4E-L). Since the effects of overexpression of Argos are ameliorated in the absence of spry function, SPRY acts either downstream or parallel to Argos. If Argos activity was mediated solely by SPRY, we would expect that removal of Argos would not affect the spry mutant phenotype. However, spry-, argos- double mutant ommatidia exhibit massive neuronal differentiation with each ommatidium containing more than a dozen neurons (Fig. 2C). Thus argos and spry are unlikely to act in series, but have parallel and partially redundant functions in antagonizing EGFR signaling.

spry can function mainly autonomously to prevent cone cells from becoming R7 cells

As SPRY appears to function as an intercellular signal in the developing tracheal system, we asked whether SPRY could act at a distance in the developing eye. Initially, we examined ommatidia located at the boundary of spry- clones in the eye. The ommatidial phenotype changed abruptly at the boundary (Fig. 8A), indicating that SPRY does not act over long distances.

To determine which cells require spry+ function for the construction of phenotypically wild-type ommatidia, we carried out a mosaic analysis on spry- clones, using a white+ transgene to mark spry+ photoreceptor neurons. Since there are no lineage restrictions during ommatidial assembly (Lawrence and Green, 1979; Ready et al., 1976; Wolff and Ready, 1991b), some ommatidia at the clone border are composed of both spry+ and spry- cells. We found that any photoreceptor cell can be genotypically spry- in normally constructed ommatidia, indicating that there is no absolute requirement for spry+ function in any of the photoreceptor neurons (Fig. 8B). However, all photoreceptor cells except R3 had a decreased probability of being spry-. This suggests that there is redundancy among photoreceptor cells in the requirement for spry+ function, and/or that there is a requirement for spry in non-neuronal cells that are related to the photoreceptor cells, such as the cone cells.

To determine whether spry is required in the cone cells that are transformed into R7 neurons, we performed a mosaic analysis on spry- clones in a sev- background. Since sev- ommatidia lack the endogenous R7 cell, all R7 cells developing in the mutant clone must arise as a consequence of the absence of spry function. In 15 retinae containing sev-, spry- mutant clones, 99 mosaic ommatidia with six outer photoreceptor cells and one or more R7 cells were scored (Fig. 8C,D). A vast
majority of the R7 cells (97.9%) were spry−, indicating that the requirement for spry+ function is mainly cell autonomous in the cells that differentiate as R7. However, three of the 140 R7 cells (2.1%) were spry+ in genotype. This percentage is significantly higher than the 0.2% expected from the dominant effect of spry in the same heterozygous genotype but in which homozygous clones were not induced. (see Methods). Thus, spry can also function non-autonomously to prevent cone cells from differentiating as R7 photoreceptor neurons.

DISCUSSION

SPRY can antagonize EGF as well as FGF signaling pathways

SPRY functions as an antagonist of the Branchless FGF signaling pathway in embryonic tracheal development (Hacohen et al., 1998). We have provided several lines of evidence that, in several other tissues, SPRY functions as an antagonist of EGFR signaling. First, spry loss-of-function mutations cause phenotypes in the developing adult eye (extra photoreceptor neurons), the larval peripheral nervous system (extra chordotonal organs) and embryonic CNS (extra midline glial cells) that closely match those caused by hyperactivation of EGF signaling pathways. Second, overexpression of spry closely mimics the effect of EGFR pathway mutations in the developing eye as well as in the developing wing and ovary. Third, spry is expressed at the same time and in close proximity to the EGF signaling pathways that it appears to antagonize in these tissues. In contrast, no FGF ligand or receptor has been implicated in the processes affected in these tissues. Although not all FGF pathway genes have been examined in each tissue, we examined the three extant ligand and receptor genes (breathless, heartless, branchless) in chordotonal organ development and found that none of the three had any detectable role. Furthermore, in the midline glial cells, where the Breathless FGF receptor is known to function, it is required for a glial cell migration process that is distinct from the glial cell survival pathway affected by spry and EGFR pathway mutations. Although we cannot exclude the possibility that spry antagonizes the EGF pathway indirectly via an effect on an unknown FGF signaling pathway, the simplest interpretation of our results is that SPRY functions as an antagonist of EGF and FGF signaling pathways in different tissues.

One of the interesting aspects of spry function in the developing trachea is that expression of the gene is induced by the signaling pathway that it inhibits (Hacohen et al., 1998). This negative feedback circuit serves to restrict the range of FGF signaling, so that only the cells located close to the FGF signaling center are induced by the FGF pathway. In the tissues examined here, there appears to be a similar coupling of spry expression and the signaling pathway that it antagonizes. In the developing eye, spry is expressed at the highest levels in photoreceptor cells located closest to the EGF signaling center and at lower levels in the photoreceptors located farther away, and loss-of-function mutations in EGF signaling pathway components result in the absence of spry expression. In the developing ovary, spry is expressed in the follicle cells directly adjacent to the Gurken EGF signaling centers in the oocyte, suggesting that, in this tissue too, EGF signaling induces expression of the SPRY inhibitor. In the developing eye, absence of spry appears to result in overactivity of the EGF pathway and neuronal induction of non-neuronal cells (mystery and cone cells) that are located beyond the normal range of the EGF inductive signal.

In all three systems in which we have shown a requirement for spry, the loss of spry function results in supernumerary neurons or glia, respectively. While the midline glia normally show some variability in the number of glial cells that escape cell death (Sonnenfeld and Jacobs, 1995), the number of photoreceptor cells per ommatidium and the number of chordotonal organs per hemisegment is invariant. This invariance is achieved, at least in part, through the tight control of induction and its response. Since spry appears to be expressed as a primary response to the signal, a reduction in the inducing signal would likely result in reduced expression of the antagonist SPRY. This feedback loop would buffer the inductive response against small changes in the levels of the inducing signal.

Argos is another extracellular antagonist of EGF signaling. Like spry, its expression is induced by the EGF pathway that it inhibits (Golembio et al., 1996). The two antagonists are expressed in similar patterns in several developing tissues including the eye, the midline glia and the ovary, suggesting that they often function together to restrict EGF signaling activity. In fact, we have shown that spry and argos exhibit a synergistic phenotype in the eye. Despite these commonalities, there are a number of significant distinctions between the two antagonists. First, in mosaic retinae, argos+ cells can rescue the argos− mutant phenotype over distances spanning several ommatidia, implying that Argos can act at long range (Freeman et al., 1992b). In contrast, SPRY action appears to be limited to the cells that express the protein or its close neighbors. Secondly, Argos appears to affect only EGF signaling, whereas our data indicate that SPRY can antagonize EGF and FGF signaling in different contexts. Thirdly, while Argos shows some limited structural similarity to EGF ligands and other proteins with EGF-like motifs, SPRY contains a novel cysteine-rich sequence motif, suggesting that the mode of action of these two proteins are distinct.

spry function in the eye has both autonomous and non-autonomous aspects

Genetic analysis of spry function in the trachea has shown that spry functions non-cell-autonomously to block FGF induction of tracheal branching: spry function is required in branching cells to prevent their non-branching neighbors from likewise elaborating secondary branches (Hacohen et al., 1998). Interestingly, our analysis of spry function in the eye indicated that spry acts mainly autonomously. We found that in most mosaic ommatidia cone cells needed to be spry− in order to aberrantly differentiate as R7 photoreceptor neurons although, in a few exceptional cases, spry+ cells differentiated as R7 cells indicating that the nearby spry− cells had influenced their development. It is possible that the autonomous action of SPRY occurs inside the spry-expressing cells, for example while SPRY is in transit to the plasma membrane. However, given the results obtained in the tracheal system, we propose that in both tissues SPRY acts as a secreted antagonist of RTK signaling but, in the eye, it has mainly autocrine effects while in the tracheal system its effects are predominantly paracrine. The differences might be due to differences in the extracellular environments of the two tissues, which might affect release or
diffusion of the protein. Alternatively, these differences might be due to differences in the pathways responsible for SPRY reception or signal transduction in the two tissues (see below), or to differences in the pathways (EGF versus FGF) that are inhibited by SPRY action.

**Implications for SPRY protein mechanism of action**

Our results have important implications for the mechanism of SPRY action. Previously, we presented three possible models of how SPRY might antagonize the Branchless FGF pathway in the developing tracheal system. One was by direct binding or blockage of the FGF ligand. Another was by binding or blockage of the FGF receptor. Breathing. A third model postulated a separate SPRY receptor on the receiving cells that antagonized the FGF pathway downstream of the FGF receptor in the receiving cells. Our data indicating that SPRY antagonizes both FGF and EGF pathways supports the third model. Because the structures of the two types of ligands (EGF and FGF) and the extracellular portions of their receptors do not show any striking sequence similarities, this argues against the first two models, which invoke direct interaction between SPRY protein with FGF or EGF ligands or receptors. However, the intracellular portions of the EGF and FGF receptors and the downstream signal transduction pathways show significant similarities. Thus, it is easy to imagine how SPRY interaction with its own receptor on a receiving cell could lead to inhibition of a common downstream step in the FGF and EGF signaling cascades. If SPRY acts through its own receptor, rather than by directly antagonizing the FGF or EGF receptors, then it is also easy to see how differences in autocrine versus paracrine activity of SPRY in different tissues could arise by differences in expression or activity of its receptor.

In summary, we have described that SPRY is a novel antagonist of EGF- as well as FGF-mediated signaling. These RTK signaling pathways therefore share not only intracellular signaling components such as the ras GT-Pase and MAPK, but also the extracellular antagonist SPRY. The ability of SPRY to regulate the activities of these two RTKs may provide a new way to allow refinement and cross-talk between these important developmental signaling pathways.

We thank the Saito, Rubin, and Goodman laboratories for antibodies and A. Brand, T. Schüpbach, K. Sawamoto, C. Klämbt, S. Paine-Saunders, B. Hays, S. Stowers, M. Simon, A. Wohliwil, S. Spencer and Kathy Matthews at the Bloomingtock Stock Center for fly strains. We thank N. Niwa and T. Nagashima for the SEM analysis and J. Goodhouse for assistance with confocal microscopy. We are grateful to S. Hayashi and T. Schüpbach for critical reading of the manuscript. S. K. wishes to thank E. Wieschaus for discussion and for his hospitality. This work was supported by grants from the Ministry of Education, Science, Sports, and Culture of Japan, JSPS (Research for the Future Program) (Y.H.), and the National Institute of Health (M.K.). M.K. is an investigator of the Howard Hughes Medical Institute.

**REFERENCES**


Klämbt, C., Glazer, L. and Shilo, B. Z. (1992). breathless, a Drosophila FGF receptor homolog, is essential for migration of tracheal and specific midline glial cells. Genes Dev. 6, 1668-1678.


Kretzschmar, D., Brunner, A., Wiersdorf, V., Pflugfelder, G. O.,