INTRODUCTION

The understanding of the molecular mechanisms by which cells respond to extracellular stimuli to proliferate or differentiate is still fragmentary. Genetic studies in organisms such as Drosophila or C. elegans, coupled with biochemistry and tissue culture experiments, have provided a partial understanding of the molecular nature of signal transduction pathways, the molecules involved and potential crosstalk between pathways (e.g. McCormick, 1989; Greenwald and Rubin, 1992; Herskowitz, 1995; Hunter, 1995; Marshall, 1995).

The small GTPase p21Ras (or Ras) performs a central function during many cellular responses, including proliferation and differentiation (e.g. Marx and Kerr, 1993; Zipursky and Rubin, 1994; Marshall, 1995). In response to signals from receptor tyrosine kinases (RTKs), Ras is activated by guanine nucleotide exchange factors (GEFs), which convert the inactive GDP-bound form to the active GTP-bound form. Activated Ras stimulates downstream effectors such as the serine/threonine kinase Raf, which is the first in a cascade of kinases, the MAPK cascade, conveying the signal to the nucleus (Marshall, 1994). Ras is inactivated by GTPase activating proteins (GAPs), which stimulate conversion to the inactive GDP-bound form (McCormick, 1989).

Ras activity is negatively regulated by GTPase activating proteins (GAPs). It has been proposed that RasGAP may also function as an effector of Ras activity. We have identified and characterized the Drosophila homologue of the RasGAP-binding protein G3BP encoded by rasputin (rin). rin mutants are viable and display defects in photoreceptor recruitment and ommatidial polarity in the eye. Mutations in rin/G3BP genetically interact with components of the Ras signaling pathway that function at the level of Ras and above, but not with Raf/MAPK pathway components. These interactions suggest that Rin is required as an effector in Ras signaling during eye development, supporting an effector role for RasGAP. The ommatidial polarity phenotypes of rin are similar to those of RhoA and the polarity genes, e.g. fz and dsh. Although rin/G3BP interacts genetically with RhoA, affecting both photoreceptor differentiation and polarity, it does not interact with the gain-of-function genotypes of fz and dsh. These data suggest that Rin is not a general component of polarity generation, but serves a function specific to Ras and RhoA signaling pathways.

Key words: RasGAP, Development, Ommatidial polarity, Ras, Rho, Drosophila

SUMMARY

The small GTPase Ras plays an important role in many cellular signaling processes. Ras activity is negatively regulated by GTPase activating proteins (GAPs). It has been proposed that RasGAP may also function as an effector of Ras activity. We have identified and characterized the Drosophila homologue of the RasGAP-binding protein G3BP encoded by rasputin (rin). rin mutants are viable and display defects in photoreceptor recruitment and ommatidial polarity in the eye. Mutations in rin/G3BP genetically interact with components of the Ras signaling pathway that function at the level of Ras and above, but not with Raf/MAPK pathway components. These interactions suggest that Rin is required as an effector in Ras signaling during eye development, supporting an effector role for RasGAP. The ommatidial polarity phenotypes of rin are similar to those of RhoA and the polarity genes, e.g. fz and dsh. Although rin/G3BP interacts genetically with RhoA, affecting both photoreceptor differentiation and polarity, it does not interact with the gain-of-function genotypes of fz and dsh. These data suggest that Rin is not a general component of polarity generation, but serves a function specific to Ras and RhoA signaling pathways.

Key words: RasGAP, Development, Ommatidial polarity, Ras, Rho, Drosophila
binds a number of other proteins (Ellis et al., 1990; Moran et al., 1991; Duchesne et al., 1993; Pomerance et al., 1996).

Increasing evidence suggests that RasGAP is itself an effector of Ras, not simply a negative regulator (reviewed by, for example, McCormick, 1989; Tocque et al., 1997). RasGAP is tyrosine-phosphorylated in fibroblast cells stimulated with EGF and in cells expressing inducible forms of v-Src or v-Fps (Ellis et al., 1990), and binds the PDGF receptor (Kazlauskas et al., 1990). Antibodies against the SH3 domain of RasGAP prevent activation of maturation-promoting factor by oncogenic Ras, without blocking the MAPK cascade (Pomerance et al., 1996) and block germlinal vesicle breakdown induced by oncogenic Ha-Ras in Xenopus oocytes without affecting Ha-Ras-GTPase stimulation by RasGAP (Duchesne et al., 1993). This suggests that Ras activates a pathway involving the SH3 region of RasGAP, in addition to the MAPK cascade.

Several proteins bind to the N-terminal region of RasGAP and are thus candidates for effectors. p190 binds to RasGAP SH2 domains in mitogenically stimulated and tyrosine kinase transformed cells. RasGAP, p190 and another RasGAP-binding protein, p62, become tyrosine-phosphorylated upon transformation of cells by cytoplasmic and/or receptor tyrosine kinases (Ellis et al., 1990; Moran et al., 1991). p190 itself has GAP activity on the Rho class of small GTPases, and in addition has homology to a transcriptional repressor (Settleman et al., 1992).

A conformational change induced by binding of p190 could modulate RasGAP activity (Hu and Settleman, 1997). Binding of two phosphorylated tyrosines on p190 to the two SH2 domains of RasGAP brings these domains in close proximity to one another, allowing the SH3 domain to bind other proteins. RasGAP SH3 binding protein (G3BP) is currently the only protein known to bind this region of RasGAP (Parker et al., 1996). G3BP binds to RasGAP only when Ras is active and is therefore an attractive candidate for an effector of Ras. Furthermore, G3BP possesses a phosphorylation-dependent exoribonuclease activity (Gallouzi et al., 1998), suggesting an involvement in the regulation of mRNA turnover in response to RasGAP-mediated signaling.

We have identified mutations in a Drosophila homologue of G3BP, encoded by the rasputin (rin) gene, and characterized its function during eye development. Phenotypic analysis indicates a role for rin in ommatidial polarity generation and in Ras signaling. Genetic interactions suggest that it functions downstream of Ras but upstream or independently of the Raf/MAPK cascade. The ommatidial polarity phenotype of rin is similar to that of the RhoA GTPase (Strutt et al., 1997). Its specific genetic interaction with RhoA suggests that it acts as a link between Ras and Rho signaling.

MATERIALS AND METHODS

Fly strains and genetics
P-element line P4957 was isolated from the EMBL lethal collection (Guichet et al., 1997). The rinM allele was generated by imprecise excision of P4957 using standard methods. Putative excision alleles were screened for female sterility over a small deletion in 87F, Df(3R)urd, since genetic studies suggested that rin was also required for fertility (C. P. and S. R. H., unpublished; rin mutants and anti-Rin antibody are available from S. R. H., e-mail: shaynes@usuhs.mil or M. M., e-mail: mlodzik@embl-heidelberg.de). DNA corresponding to the rin locus was analyzed by PCR and genomic Southern blotting. One line, rin*, had a deletion of the entire coding region. The deletion extended for approx. 14 kb and removed most of the P-element, the nearby Tsr gene and part of the Hrb87F gene (Haynes et al., 1997).

The tub-rin transgene was generated by inserting a 3.2 kb cDNA containing the complete ORF into pCaSpeR downstream from a 2.4 kb fragment containing the tubulin promoter. Transgenic lines were obtained by standard procedures (Spradling and Rubin, 1982). One copy of this construct rescued the eye phenotype of several allelic combinations. UAS-rin was generated by inserting the same 3.2 kb cDNA into pUAST (Brand and Perrimon, 1993); transformant lines were crossed to various GAL4 driver lines.

The following Ras signaling pathway strains were used for genetic interaction crosses: sev111 (Basler et al., 1991), sev18, Sos1C2 (Rogge et al., 1991), sevRaf1 (Karim et al., 1996), sevRaf1 (Dickson et al., 1992), rtdEm (Brunner et al., 1994), Gap116 (Gaul et al., 1992), sevGAL4, UAS-Gap1 (gift of U. Gaul), sev-Jun40 and sev-Jun64 (Treier et al., 1995) and sevGAL4, UAS-RhoA (Strutt et al., 1997).

Histological analysis
Eye sections and antibody stainings of eye discs were performed as described (Tomlinson and Ready, 1987). The following antibodies were used: rat anti-ELAV (gift from G. Rubin), rat anti-Sal-r (gift from R. Barrios), rabbit anti-Bar (gift of K. Saigo), mouse anti-β-gal (Promega), all at 1:200 dilution, and fluorescently labelled secondary antibodies (Jackson Labs). Embryos were fixed and stained as described (Patel, 1994), with rabbit anti-Rin (diluted 1:500; 1:200 for discs), and an HRP-coupled donkey anti-rabbit secondary antibody (diluted 1:100, from Amersham). Pictures were processed with Adobe Photoshop.

Molecular techniques
Genomic DNA adjacent to the P-element was isolated by plasmid rescue. Using the corresponding fragment as a probe, cDNAs were isolated from a whole disc cDNA library (gift from K. Basler), and using portions of a rin (Nts) cDNA from R. Kelley (Kelley, 1993) as a probe from an ovariain cDNA library (Steinhauer et al., 1989). Representitive cDNAs from both libraries and the cDNA from R. Kelley were sequenced by standard molecular methods. Portions of a rin cDNA encoding amino acids 1-171 and 494-589 (corresponding to the conserved N-terminal domain and the RRM, respectively) were amplified by PCR using Vent DNA polymerase (NEB) and cloned separately into pET-28a (Novagen). Clones were verified by sequencing. Recombinant proteins were produced and purified on His-Bind resin (Novagen) as described by supplier. Purified proteins were used to generate rabbit polyclonal antiserum. Antiserum against both proteins recognized the same size band on western blots; the anti-RRM antiserum gave a stronger response, and was used for all subsequent experiments.

For western blotting, samples were resolved on 8%-16% Tris-glycine gradient gels (Novex) and transferred to nitrocellulose using a Novex transblot apparatus. Blots were blocked in PBS, 0.05% Tween 20 and 5% nonfat dry milk, and immunoblotted with anti-Rin antibody diluted 1:5000 in PBS, 0.05% Tween 20, then incubated with HRP-conjugated donkey anti-rabbit secondary antibody (Amersham) diluted 1:50,000 in PBS, 0.05% Tween 20. Signals were detected with enhanced chemiluminescence.

RESULTS
Isolation of a mutation in rasputin as a dominant suppressor of sev-svp
The nuclear receptor Seven-up (Svp) is expressed in and required for the correct specification of a subset of outer
photoreceptors in the developing eye (Mlodzik et al., 1990). Ectopic expression of svp in any photoreceptor precursor or in cone cells (svp-svp) causes cell fate transformations and a rough eye phenotype that is dosage-sensitive (Hiromi et al., 1993). Loss-of-function mutations in all components of the Ras/MAPK pathway act as dominant suppressors of this phenotype, whereas negative regulators of the pathway act as dominant enhancers (Begemann et al., 1995; Kramer et al., 1995). To identify other components involved in Ras signaling during photoreceptor determination or, more specifically, genes required for outer photoreceptor specification we used a svp-svp strain to screen for second site modifiers. A collection of approximately 4,500 P-element insertions on the second and third chromosomes (Guichet et al., 1997) was screened (not shown). Strain P4957 was identified as a dominant suppressor. The single P-element is inserted at chromosomal band 87F3-6 within a known transcription unit adjacent to the squid gene (originally referred to as Next-to-squid; Kelley, 1993). We have named this gene rasputin (rin) and this P-allele is referred to as rin1. Both the homozygous phenotype of rin1 (see below) and the dominant suppression of svp-svp are revertible upon excision of the P-element (see Materials and Methods).

To generate additional alleles of rin we mobilized the P-element and selected for excision events. PCR and Southern analysis of the rin2 allele showed that the entire open reading frame had been deleted; thus rin2 is a null allele (see Materials and Methods). Homozygous or transheterozygous rin1 and rin2 flies are viable and exhibit a mild rough eye phenotype. When transheterozygous to a small deficiency uncovering the region rin encodes the Drosophila G3BP homologue.

rin is required for photoreceptor recruitment and ommatidial rotation

Detailed analysis of the eyes of rin mutant alleles revealed defects in two aspects of eye development. Externally such eyes are mildly rough (not shown) with defects in both photoreceptor recruitment and ommatidial polarity, as apparent in sections (Fig. 1). Many ommatidia have fewer than the wild-type complement of eight photoreceptors, and some have extra photoreceptors (Fig. 1E). Both inner and outer photoreceptors can be affected, indicating a general role for rin during photoreceptor specification.

Many rin mutant ommatidia with the normal photoreceptor complement display polarity defects (Fig. 1), reminiscent of the phenotypes of tissue polarity mutations such as frizzled (Adler, 1992; Zheng et al., 1995). In wild type the arrangement of photoreceptors in ommatidia in the ventral and dorsal fields are mirror images (Ready et al., 1976). This polarity is established in the imaginal disc, concurrent with cell fate specification. Developing ommatidial preclusters begin to rotate in opposite directions on either side of the dorso-ventral midline, the equator, finally reaching an angle of 90° relative to their starting position. At the same time the R3/R4 precursors move into asymmetric positions, giving rise to ommatidia of opposite chirality on either side of the equator (Fig. 1A; Ready et al., 1976; Reifegeste and Moses, 1999; Mlodzik, 1999). rin affects all aspects of this process: the direction and degree of rotation, and the generation of the chiral forms (see Fig. 1).

To analyze these defects during early development we examined rin mutant eye discs. Both the recruitment and polarity defects are evident in third instar imaginal discs (Fig. 2), indicating an early requirement for rin in both processes. Developing photoreceptor cells are often missing in mutant discs (as visualized with anti-Elav, anti-Bar or anti-Sal staining, Fig. 2). Similarly, the wild-type arrangement and orientation of ommatidial preclusters (Fig. 2C) is affected (Fig. 2B,D), indicating that these are primary defects.

In addition to the eye defects (Figs 1 and 2), most rin mutant adults hold their wings out and null mutants are sterile (not shown; will be described elsewhere). It is not clear whether rin is required for embryonic or early larval functions. Northern and western analyses (see below) indicate that large amounts of maternal rin mRNA and protein are deposited in the egg, and this may be sufficient for early development.

rin encodes the Drosophila G3BP homologue

Genomic DNA flanking the P-element insert in rin1 was isolated (see Materials and Methods) and used to screen an imaginal disc cDNA library. Seven independent overlapping cDNAs were recovered. Also, the original rin cDNA (referred to as Nts; Kelley, 1993) was used to isolate cDNA clones from an ovarian library. The complete nucleotide sequence of the cDNAs and the exon-intron boundaries within the genomic DNA were determined (Fig. 3A). Multiple rin transcripts were detectable at all stages during development (Fig. 3C). The highest transcript levels were seen in 0-3 hour embryos, suggesting a maternal contribution to the embryo (Fig. 3C). The different transcripts result from the use of multiple polyadenylation sites within the 3¢ untranslated region (UTR) and the use of an alternative exon in the 5¢ UTR. Comparison of the sequence surrounding the P insertion with those of the cDNAs revealed that the P-element is inserted within the alternative exon in the 5¢ UTR.

Conceptual translation of the ORF present in the cDNAs predicts a protein of 690 residues (Fig. 3B), sharing extensive homology with the vertebrate p120 RasGAP SH3 binding protein (G3BP) (Parker et al., 1996). Mammalian G3BP was isolated due to its ability to bind the SH3 domain of p120 RasGAP in ER22 cells when Ras is in its active, GTP-bound form. Two forms of G3BP, differing mostly in their central domains, are found in humans and mice, and a homolog has been found in S. pombe. Rin shares 40% amino acid identity and 60% homology with human G3BP1 and G3BP2 over their entire lengths (Fig. 3B) and is therefore likely to be the Drosophila homologue of G3BP. The S. pombe protein is less closely related to the others (26% identity and 36% homology to Rin), but the sequence conservation is significant. The proteins can be divided into four domains, which are conserved to different extents. Highest conservation, suggesting an important role, is found in the N terminus in a region with no predicted function. Each protein has a moderately well-conserved RNA recognition motif (RRM). RRM-type RNA binding domains are present in many known RNA binding proteins (Birney et al., 1993). The RNA-binding surface of the RRM is a four stranded β-sheet; residues in adjacent loops may help to recognize specific RNA sequences. The RRM in Rin has two short inserts relative to the human G3BP proteins; these are predicted to be within loop regions (Birney et al., 1993). An arginine/glycine-rich (RGG) domain is located at the C terminus of human and Drosophila Rin.
RGG sequences, which have been implicated in RNA binding (Kiledjian and Dreyfuss, 1992), are found in the human proteins. The central portion of the protein is the most divergent, both between and within species. This acidic, proline-rich region contains the SH3 binding domain in G3BP, and this region consists mostly of proline and glutamine residues. Although we do not know whether Rin binds an SH3 domain, it contains several PXXP motifs (double overlining in Fig. 3B), which are characteristic of SH3-binding proteins (Feller et al., 1994).

To confirm that the eye phenotypes described above (Figs 1, 2) are due only to lesions in rin, we expressed a full-length cDNA under the control of the tubulin promoter (tub-rin) and asked whether this transgene can rescue these phenotypes. The eye phenotypes of both rin alleles and transheterozygous
animals were fully rescued (Fig. 1F and not shown), indicating that this cDNA encodes the gene affected in the mutants.

**Rin is cytosolic and localised apically in photoreceptor precursors**

We cloned sequences encoding the Rin RRM domain into an expression vector and generated polyclonal antisera (see Materials and Methods). Rin is expressed throughout development (Fig. 4A). Consistent with the abundant maternal transcripts, Rin is highly expressed in early embryos (Fig. 4A,B). In *rin* adults and eye imaginal discs protein levels are significantly reduced as compared to wild type (compare Fig. 4A, lanes 7 and 6, and Figs 4E,F with 2A,D). Rin is cytosolic and excluded from the nucleus in all tissues analyzed (Fig. 4 and not shown). In addition, Rin is localised apically in the developing cells in the eye imaginal disc (Fig. 4D), similar to...
Fig. 4. Expression of Rin. (A) Western blot analysis of Rin. Lanes 1-5, developmental expression profile: (1) 0-12 hour embryo, (2) 1st and 2nd instar larvae, (3) early pupae, (4) late pupae, (5) adult fly. A protein of about 75 kDa is recognized, in agreement with the predicted size (74895). Lanes 6-9, Rin expression in adult flies of the following genotypes: (6) wild type, (7) rin1, (8) rin2, (9) rin2/Df(3R)rad. Rin protein is reduced in rin1 and absent in rin2 flies. Balanced loading was verified by determining protein concentration (Biorad assay) and staining the blot with Ponceau S before antibody incubation. Each lane contains total protein equivalent to one whole adult male. (B) Rin embryonic expression, detected with an HRP-coupled secondary antibody. Note the ubiquitous cytoplasmic expression. (C-F) Immunofluorescence images of a wild-type 3rd instar eye imaginal discs. Rin (green channel) and Elav (C,F) or BP104 (D) are shown (red channel; marking all photoreceptors). Rin is expressed in all cells of the disc (C) including cells of the peripodial membrane; (E,F) at higher magnification show the cytoplasmic localisation of Rin (E), which is excluded from the nuclei (overlay with Elav staining, F). An XZ-section through a disc (D) highlights the subcellular apical concentration of Rin.

the localisation of Ras pathway proteins such as Drk, Sos and Dos (Olivier et al., 1993; Karlovich et al., 1995; Raabe et al., 1996; see below).

Overexpression of Rin causes defects in ommatidial polarity and photoreceptor recruitment
To further characterize the role of rin during eye development, we overexpressed Rin in the developing eye using the GAL4/UAS system (Brand and Perrimon, 1993). UAS-rin was expressed under the control of the sevenless-GAL4 driver (in R3/4, R1/6, R7, the mystery and cone cells; Basler et al., 1989) (Fig. 5). At 25°C such eyes showed extensive roughening, and photoreceptor recruitment was affected in many ommatidia (Fig. 5B); there were missing and extra photoreceptors of both the inner and outer sub-types. In ommatidia in which orientation can be scored (those with the normal photoreceptor complement) polarity is severely affected. Many ommatidia are misrotated and/or display a non-chiral arrangement. In addition, defects in rhodomere morphology are often present. Consistent with the temperature sensitivity of GAL4-driven expression, sevGAL4, UAS-rin flies raised at 18°C had less severely rough eyes, and all phenotypic aspects are weaker in flies raised at 18°C (Fig. 5A). In such eyes the equator is still evident, with mostly the correct chiral forms in each eye field, but many ommatidia are misrotated (Fig. 5A).

To test whether these phenotypes are primary defects, we analyzed sevGAL4, UAS-Rin eye discs. The recruitment and polarity defects are evident in early third instar discs (Fig. 6; visualized with anti-Elav and anti-β-gal in an svp-lacZ line; svp is expressed in R3/R4 and later also in R1/R6; Mlodzik et al., 1990; Fanto et al., 1998). Developing photoreceptor cells are often missing (examples are indicated with arrowheads) and the regular wild-type arrangement and orientation of ommatidial preclusters in mutant discs is affected (as visualized with svp-lacZ, Fig. 6). These data indicate that these phenotypes are primary defects.

The observation that the loss- and gain-of-function polarity phenotypes of rin are similar is reminiscent of the tissue polarity genes, where either loss of a protein or overexpression

<table>
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<tr>
<th>Genotype</th>
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<tr>
<td>sev511</td>
<td>Su</td>
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<td>sevE4;SosC2</td>
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<td>sevRasV12</td>
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<td>rps6</td>
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<tr>
<td>Gap11-16</td>
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<td>sevGAL4, UASGap1</td>
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Su, suppression; –, no visible interaction; n.d., not done.

Flies were heterozygous for the indicated rin mutation and the dosage-sensitive gene of interest, from crosses grown at constant temperature. On average 3-7 sectioned eyes were analyzed statistically for each genotype. Mutations in rin dominantly suppress activated sevless (sev511); average number of R7s per ommatidium in sev511/+ is 3.38±0.26 (mean ± s.d, n=6 eyes), in sev511, rin1 is 2.71±0.23 (n=7 eyes) and in sev511; rin2 is 2.32±0.11 (n=5 eyes). rin mutations relieve the suppression of R7 loss in sevE4;SosC2.

The percentage of ommatidia with an R7 cell is 28.7% in sevE4; SosC2/+ (n=411 ommatidia); 13.9% in sev511; SosC2/+; rin1/+ (n=293 ommatidia) and 18.2% in sevE4; SosC2/+; rin2/+ (n=545 ommatidia).

1The sevRasV12 allele used was CR2 (Karim et al., 1996); suppression of a different sevRasV12 alleles was also observed. No statistical analysis was possible with these genotypes as the unsuppressed phenotype is too severe to allow photoreceptor counting.

2No suppression of sevRafV19 was seen either externally or upon statistical analysis.

3A very weak, statistically insignificant, effect on rps6 was observed upon analysis of sections [average number of R7s per ommatidium 3.47±0.24 in rps6+/+ (n=3 eyes) as compared to 3.13±0.22 in rps6+/+ (n=3 eyes)] and 3.42±0.3 in rps6+/+; rin1/+ (n=3 eyes)]; no suppression was obvious externally.

No interaction was observed with either Gap11-16 or with the phenotype caused by overexpression of Gap1, sevGAL4, UASGap1. No interaction was observed with any of the putative nuclear targets of Ras signaling.
cause similar defects (Krasnow et al., 1995; Strutt et al., 1997). Similarly, the rhabdomere morphology defects are also present in the stronger RhoA gain- and loss-of-function alleles (Strutt et al., 1997). The photoreceptor recruitment defects observed in the gain-of-function situation are consistent with a role in Ras-mediated photoreceptor induction (see below).

**Mutations in rin genetically interact with Ras and Rho signaling**

The high homology of Rin to vertebrate G3BPs, the defects in photoreceptor recruitment and the subcellular apical enrichment of Rin (Fig. 4D) suggested an involvement of rin in Ras signaling. To test this further we analyzed genetic interactions between Ras pathway components and rin. Constitutive activation of Ras signaling components during eye development, by expression of activated alleles under the control of the sev-enhancer, causes the recruitment of ectopic photoreceptors (mainly a transformation of cone cell precursors to R-cells) and an externally rough eye (Basler et al., 1991; Dickson et al., 1992; Fortini et al., 1992; Brunner et al., 1994). Reduction of the dose of a gene required for this cell-fate transformation often reduces the number of extra photoreceptors and visibly alters the phenotype. This approach has been used successfully in genetic screens for downstream targets of known components of Ras signaling (reviewed in Zipursky and Rubin, 1994; Wassarman et al., 1995; Freeman, 1997).

Strikingly, rin interacts with those members of the pathway that function at the level of Ras and above and not with downstream components such as Raf (Fig. 7, Table 1). Loss of one copy of rin dominantly suppresses the phenotypes caused by the sevenless RTK (sevS11) and sevRasV12 (Fig. 6A,B and C-F, respectively), and relieves the partial rescue of sevE4 by SosJC2 (Table 1), a gain-of-function mutation in the GEF (Rogge et al., 1991). In contrast, rin dosage does not affect the phenotype caused by activated Raf (sevRafY9) or other downstream components analysed (Table 1). Additionally, there is no dosage effect of rin mutations on the phenotype caused by mutations in Gap1 or on the phenotype caused by overexpression of Gap1 (sevGAL4, UAS-Gap1; our unpublished data). These data support the notion that Rin functions in the context of Ras signaling. The specificity of the interactions suggests that Rin acts upstream of or in a parallel pathway to Raf (see Discussion).

The defects in ommatidial polarity in rin mutants and upon Rin overexpression suggested an involvement of Rin in planar polarity (Fz/Dsh) signaling. Polarity specific gain-of function genotypes such as sev-Fz and sev-Dsh interact genetically with other components of planar polarity signaling (Strutt et al., 1997; Boutros et al., 1998). Also it has been shown that Notch (N) signaling plays a specific role in polarity establishment in the eye downstream of Frizzled (Cooper and Bray, 1999; Fanto and Mlodzik, 1999). To ask whether rin interacts genetically with these genotypes, we tested whether rin mutations affect the sev-Fz, sev-Dsh, sev-N* and sev-RhoA phenotypes. Whereas rin mutations do not affect the gain-of-function polarity phenotypes of fz, dsh and N (neither externally nor in sections, not shown), they dominantly enhance the eye phenotype caused by overexpression of wild-type and activated RhoA (sev-RhoA and sev-RhoAV14; Fig. 8 and not shown). rin mutants affect both aspects of the RhoA phenotype, photoreceptor differentiation and polarity generation. These data suggest that rin is not a common component of polarity signaling but appears to have a general requirement for RhoA function.

**DISCUSSION**

We have characterized the rasputin (rin) gene, encoding the
Drosophila homologue of vertebrate G3BP, a p120 RasGAP SH3 domain binding protein. Mutations in rin lead to defects in eye development, affecting both photoreceptor recruitment and ommatidial polarity, resembling phenotypes of Ras1 and RhoA mutants. These observations and genetic interaction data support a role for Rin in Ras-mediated signaling. Mutations in rin dominantly suppress phenotypes caused by activated components of the Ras pathway, acting at the level of Ras and above. We originally isolated a rin allele in a screen for genes interacting with sev-svp (Begemann et al., 1995), thus this interaction is consistent with a role for rin in Ras signaling.

Rin as a potential Ras effector
Ras activation is required for the development of all photoreceptors (for reviews see Wassarman et al., 1995; Freeman, 1997). The cytoplasmic components of Ras signaling colocalise apically in differentiating photoreceptors (Olivier et al., 1993; Karlovich et al., 1995; Raabe et al., 1996). We find that Rin/G3BP is also enriched apically, consistent with a role in Ras signaling.

The Drosophila GAP, Gap1, negatively regulates Ras during eye development (Gaul et al., 1992). However, Gap1 does not contain the SH2-SH3-SH2 domains found in the vertebrate p120 RasGAP. Therefore if Rin associates with the SH3 domain of RasGAP, it does so with a GAP protein other than Gap1. Consistent with this, our genetic studies provide no evidence for an interaction between rin and Gap1: rin mutations affect neither the Gap1 mutant phenotype nor that caused by Gap1 overexpression. A candidate for another GAP is the recently described Drosophila RasGAP gene, which contains an SH2-SH3-SH2 region similar to that found in the mammalian protein (Feldmann et al., 1999). Mutations in this gene are not currently available, however, and thus the role of this RasGAP remains to be elucidated.

Our in vivo analyses suggest a number of possible
scenarios for how Rin/G3BP is involved in Ras signaling. First, the association of Rin with RasGAP could simply interfere with the catalytic RasGAP activity on Ras. Alternatively, Rin could be an effector of a signal that comes from Ras via RasGAP and is required for Raf activation. Our data support a more complex third model in which Rin would act as an effector of Ras via RasGAP in a manner that is independent of the Raf/MAPK cascade (Fig. 9). We speculate that Rin binds the SH3 domain of RasGAP when Ras is active (as does human G3BP) and exerts an effect itself, or it interferes with the association of RasGAP with other effector proteins. This model is supported by the data: (1) rin does not interact with the Raf/MAPK cascade downstream of Ras, yet does suppress the effect of Ras activation; (2) RasV12 is insensitive to GAP activity (John et al., 1988) and thus rin should not have an effect on sevRasV12 if its only effect was on RasGAP catalytic activity; (3) both Ras and Raf are required for proliferation of imaginal disc cells, whereas rin− tissue proliferates. Although our results do not rule out any of these models, we favour the last model, because of the association of G3BP with RasGAP in mammalian cells (Parker et al., 1996), the effector function proposed for RasGAP and the above mentioned differences in Raf and Rin requirements.

The role of Rin in Rho signaling
Mutations in rin and RhoA (Strutt et al., 1997) cause similar defects during eye development, and they interact genetically. The enhancement of RhoA gain-of-function by rin is very specific (no other gene tested interacted with RhoA) and affects both aspects of the RhoA phenotype, photoreceptor differentiation and planar polarity. Moreover, rin does not interact with other genotypes tested, including the sev-Fz and sev-Dsh polarity specific phenotypes, and sev-N*, which affects both photoreceptor recruitment and polarity (Fortini et al., 1993; Cooper and Bray, 1999; Fanto and Mlodzik, 1999). Thus, the interactions of rin with Ras and RhoA are very specific and restricted to these factors.

Could these interactions share a common reason? Polarity determination and ommatidial rotation occur concurrently with photoreceptor recruitment. The respective signals are being transduced in the same cells within a short period of time. It is therefore conceivable that some genes are involved in both pathways or are necessary to insulate them from each other. RhoA itself is required for both processes: strong alleles show defects in photoreceptor recruitment and differentiation, and polarity (Strutt et al., 1997). Although components of Ras signaling do not interact with the Fz/Dsh polarity pathway (Strutt et al., 1997; Boutros et al., 1998), it remains possible...
that some aspects of Ras signaling might have an influence on the rotational aspect of planar polarity.

What could be the molecular mechanisms? Vertebrate p190 that binds to the SH2 domains of p120 RasGAP is itself a GAP acting on Rho/Rac GTPases (Settleman et al., 1992). The involvement of Rin in polarity establishment may therefore arise from a positive or negative interaction with a p120/p190 protein complex (Fig. 9). Rin might be required for the action of p190 on RhoA, or may interfere with binding of other proteins required in this process. Although no interaction has been detected between Ras and RhoA (not shown), rin appears to have a role in both Ras and Rho signaling, and might act as a link between these two signaling molecules.

In this context it will be interesting to determine whether Rin binds RNA, as suggested by its homology, and how this could affect Ras or RhoA signaling. Rin contains motifs found in RNA binding proteins: an RRM and an arginine-glycine-rich region (Birney et al., 1993). The effector function of Rin could therefore be in regulating trafficking, stability or translation of RNAs encoding factors required during Ras/Rho signaling. Other putative RNA binding protein are also implicated in signal transduction. HnRNP-K, found in complexes with nuclear pre-mRNA (Matunis et al., 1992) and also involved in transcriptional regulation of c-myc (Michelotti et al., 1996), binds to the SH3 domains of p95\textsuperscript{av} and Src (Hobert et al., 1994; Taylor and Shalloway, 1994). Src also associates with another RNA binding protein, p68 (Taylor and Shalloway, 1994). Thus RNA binding proteins may emerge as more general effectors of signal transduction.

We are most grateful to R. Kelley and U. Gaur for sharing unpublished materials and information. We thank R. Barrios, K. Basler, U. Gaul, E. Hafen, F. Kafatos, G. Rubin and U. Weber for fly stocks and reagents, R. Kelley for the genomic map and cDNA clone. We are grateful to Ann-Mari Voie for embryo injections, Anna Czyklaft for chromosome in situ, and Monica Cooper for technical assistance. C. Blaumueller, T. Bouwmeester, L. Kockel, N. Paricio, K. Weigmann and U. Weber made helpful comments on the manuscript. C.A.M was supported by a grant from the Boehringer Ingelheim Fonds.

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