Characterization of rho GTPase family homologues in *Drosophila melanogaster*: overexpressing *Rho1* in retinal cells causes a late developmental defect

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The rho family of GTPases has been implicated in regulating changes in cell morphology in response to extracellular signals. We have cloned three widely expressed members of this family from *Drosophila melanogaster*; a rho homologue (*Rho1*) and two rac homologues (*Rac1* and *Rac2*). Flies harbouring a *Rho1* transgene that is specifically expressed in the eye exhibit a dramatic dose dependent disruption of normal eye development. Flies bearing at least two copies of the transgene display a severe rough eye phenotype characterized by missing secondary and tertiary pigment cells, a substantial reduction in the number of photoreceptor cells and a grossly abnormal morphology of the rhabdomeres. Cell fate determination in the imaginal disc occurs normally and abnormalities become manifest late in pupariation, coincident with the phase when the cells undergo major morphological changes. This phenotype is modified by mutations at several other loci that have been implicated in signal transduction, but not by mutations in ras pathway components.

Key words: *Drosophila*/eye/GTPase/rac/rho

Introduction

The steadily growing family of small GTPases has been implicated in a wide variety of cellular signalling pathways that mediate proliferation and differentiation, cytoskeletal organization, protein transport and secretion (Bourne *et al.*, 1990; Hall, 1990). The ras GTPase, which plays a role in a large number of human cancers, has been by far the best studied of these and has served as a useful model for study of GTPase regulation and function. Through a combination of biochemical studies in mammalian systems and genetic approaches, particularly in *Drosophila melanogaster* and *Caenorhabditis elegans*, several protein components of the ras signalling pathway have now been identified (reviewed by Greenwald and Rubin, 1992; Dickson and Hafen, 1993). These include GTPase activating proteins (GAPs) (Gaul *et al.*, 1992), nucleotide exchange promoting factors (Simon *et al.*, 1991; Bonfini *et al.*, 1992), adaptor molecules (Olivier *et al.*, 1993;

Simon *et al.*, 1993) and downstream kinases (Dickson *et al.*, 1992; Tsuda *et al.*, 1993; Biggs *et al.*, 1994) that may serve as effectors of the activated ras GTPase. The identification and ordering of such pathway components has allowed the establishment of a linear ras-mediated signalling cascade that is initiated by the interaction of an extracellular ligand with its cognate cell surface receptor and presumably results in modification of the nuclear transcription pattern and phenotypic changes in the stimulated cell.

A second, less well-characterized family of GTPases is the rho family, which includes the *rho*, *rac* and *CDC42* genes (Hall, 1990). Members of this family have been identified in organisms ranging from yeast to humans, indicating that, like the ras proteins, the rho GTPases have been well conserved throughout evolution. While the function of these GTPases has not been clarified, it appears that the rho proteins play a role in regulating cell morphology. It has been demonstrated in fibroblasts by microinjection that the rho A protein regulates the assembly of focal adhesions and of the actin cytoskeleton and that rac 1 protein induces membrane ruffling (Ridley and Hall, 1992; Ridley *et al.*, 1992). These same changes can be induced in cells by the addition of extracellular growth factors and the rac and rho proteins mediate these responses. While rho family GTPases do not exhibit the focus-inducing activity observed with ras GTPases, they have been implicated in the oncogenic process (Avraham and Weinberg, 1989; Hart *et al.*, 1991; Habets *et al.*, 1994). Additionally, rho-mediated morphologic effects can be induced by activated ras protein, suggesting that ras and rho proteins might lie in the same signalling cascade. Possibly, such a connection ensures that rho-mediated morphologic changes are appropriately coordinated with ras-mediated proliferation and differentiation events. While the mechanism by which these distinct signalling pathways might be integrated is not known, the reported complex between p120 rasGAP and p190 rhoGAP may play a role (Settleman *et al.*, 1992).

The demonstration that the rho and rac GTPases can regulate cell morphology in response to extracellular signals provides a starting point for studying the mechanisms that regulate cell shape changes during the normal development of an organism. The identification of rho and rac homologues in *Drosophila melanogaster* would facilitate an analysis of their function *in vivo* using genetic techniques. Here we report the isolation and characterization of rho and rac GTPase cDNA clones from a *Drosophila* cDNA library. The *rho* and *rac* homologues appear to be expressed widely during embryonic development. Flies that overexpress a *Rho1* transgene specifically in the developing eye exhibit a severe dose dependent disruption of eye development, although cell-fate determination in the imaginal disc appears to be unperturbed.

The effects of the *Rho1* transgene can be modified by mutations at several other loci, although not by various mutations in genes whose products function in the ras-mediated signalling pathway.

Results

Isolation of cDNA clones encoding *Drosophila rho* family homologues

The rho family GTPases, which include rho, rac and CDC42, are highly conserved evolutionarily; highly related *rho* and *CDC42* genes have been identified in yeast and in mammals. Based on the reported sequences for several rho family GTPases in several species we designed degenerate oligonucleotides corresponding to the most conserved regions of these proteins. These regions include the GDGA(C/V)GKT sequence, the PTVFENY sequence and the WDTAGQ sequence, each of which is well conserved amongst the reported *rho* family genes. To isolate fragments of *Drosophila* genes that encode rho family GTPases, these oligonucleotides were then used in a 'low stringency polymerase chain reaction (PCR)' (see Materials and methods) with *Drosophila* genomic DNA as template. PCR products were purified and then sub-cloned into pBluescript and sequenced. Of the 12 clones that were sequenced, five appeared to represent partial *rho*-related fragments based on their similarity to known *rho* sequences (data not shown). Of those five, four were highly related to *rac* sequences and one was closely related to known *rho* sequences. Cloned DNA inserts isolated from one *rac* clone and one *rho* clone were mixed, radiolabelled and used as probes to detect hybridizing cDNA clones from a *Drosophila* eye—imaginal disc cDNA library. Several clones were identified that gave positive signals upon secondary and tertiary hybridization. Sequencing the cloned inserts of these cDNAs and comparison of the sequences with those of previously reported *rho* and *rac* genes revealed that one *Drosophila rho* homologue and two *Drosophila rac* homologues had been isolated (Figure 1). These clones are referred to as *Rho1* and *Rac1* and *Rac2* respectively. The cDNAs each contain an open reading frame of the expected size in addition to 5' and 3' untranslated sequences.

The predicted coding sequences of the *Drosophila* cDNAs exhibit substantial sequence similarity to their human counterparts (Figure 2A). *Drosophila Rho1* is 86% identical to the human rhoA, B and C GTPases at the amino acid level, with the vast majority of the differences occurring in the C-terminal third of the protein. The *Drosophila Rho1* protein also exhibits substantial sequence identity (~75%) with two yeast rho GTPases that have been reported, Yrho1 and Yrho2, indicating that the *rho* gene has been well maintained throughout evolution.

The *Drosophila Rac1* and *Rac2* GTPases are very similar to each other (92% identity), with most of the differences occurring at the C-termini of the proteins (Figure 2B). These GTPases are about 91% identical to two reported human rac sequences, 85% identical to a rac sequence isolated from *C.elegans* and 67% identical to a rac GTPase from the garden pea. As in the case of the rho GTPase, most of the amino acid differences occur in the C-terminal region of the protein.

The locations of each of these genes on the cytogenetic

map have been determined. *Rho1* maps to 3C, while *Rac1* and *Rac2* map to 61F and 66A respectively (data not shown). Each of the probes hybridized to a single site on the polytene chromosomes.

Expression of rho family GTPases is widespread during embryogenesis

To determine the pattern of expression of each of these genes during embryogenesis, *in situ* hybridization analysis was performed at various stages of development with single-stranded *Rho* and *Rac* anti-sense RNA probes (Figure 3A–C, E–G and I–K). Sense probes were used as negative controls (Figure 3D, H and L). The *Rho1*, *Rac1* and *Rac2* genes are each expressed throughout embryogenesis in a widespread manner. *Rac2* and *Rho1* RNA is detectable at relatively uniform levels throughout the embryo. Although widely expressed, the *Rac1* gene appears to be expressed at particularly high levels in certain tissues. Following gastrulation in germ band-extended embryos (Figure 3F), expression is highest in the mesoderm and the central nervous system and in the primordium of the hindgut. At later stages (Figure 3G) expression is more uniform.

Overexpression of *Rho1* disrupts eye development in a dose dependent manner

Tight regulation of ras-mediated signals appears to be crucial for normal eye development in *Drosophila* (Simon *et al.*, 1991; Fortini *et al.*, 1992; Gaul *et al.*, 1992). In light of the apparent connections between the ras and rho pathways and the role of rho GTPases in regulating cell morphology, we chose to examine the possibility that increased *Rho1* activity in the *Drosophila* eye might perturb the normal programme of development. The *Drosophila* compound eye is composed of ~750 units or ommatidia (Wolff and Ready, 1993) and develops from an epithelial monolayer, the eye—imaginal disc. During the third instar of larval development, cells in the imaginal disc which are posterior to the morphogenetic furrow, a transverse indentation which moves anteriorly across the disc during late larval development, are progressively recruited to developing ommatidial clusters and are instructed by their neighbours to adopt specialized fates, such as those of photoreceptor and cone cells. During the pupal phase of development, the pigment cells are determined and then all the cells undergo a series of morphological changes; the retina increases in thickness and the photoreceptor cells elaborate the rhabdomeres which contain the rhodopsin.

To determine the effect of overexpressing *Rho1* in cells of the developing eye, transgenic flies were established that carry a wild-type copy of the *Rho1* gene in the transformation vector pGMR (Figure 4A) (Hay *et al.*, 1994). pGMR is a modified P element vector that contains five copies of a binding site for the transcription factor Glass located upstream of a TATA element (see Materials and methods). Expression from this promoter is initially restricted to all the cells of the eye—imaginal disc that are located posterior to the morphogenetic furrow (Ellis *et al.*, 1993) and is maintained during later stages of eye development.

Five independent transgenic lines, *GMR-Rho1*^{1–5}, were established and the transposon integrations were mapped

Fig. 2. Alignment of *Drosophila* Rho1, Rac1 and Rac2 predicted proteins with homologues from other species. Amino acid differences are highlighted in black. (A) the *Drosophila* Rho1 protein is compared with the human RhoA, B and C proteins (Yeramian *et al.*, 1987; Chardin *et al.*, 1988) and the Rho1 and 2 proteins from the yeast *Saccharomyces cerevisiae* (Madaule *et al.*, 1987) (B) the *Drosophila* Rac1 and Rac2 proteins are compared with *C.elegans* Rac1 (Chen *et al.*, 1993), human Rac1 and Rac2 (Didsbury *et al.*, 1989) and a homologue from the garden pea called Rho1P (Yang and Watson, 1993). Residues that differ between the two *Drosophila* Rac proteins are indicated with an asterisk.

Fig. 3. Expression of *Rho1*, *Rac1* and *Rac2* in *Drosophila* embryos. Antisense probes were used to detect transcripts of *Rho1* (A–C), *Rac1* (E–G) and *Rac2* (I–K). Control hybridizations were with sense strand probes for *Rho1* (D), *Rac1* (H), *Rac2* (L). Blastoderm embryos are shown in (A), (E) and (I); germ band-extended embryos in (B), (D), (F), (H), (J) and (L); germ band-retracted embryos are shown in (C), (G) and (K). For all the embryos anterior is to the left and dorsal is towards the top of the figure.

described for several other *Drosophila* genes (reviewed by Wu and Goldberg, 1989; Pirota, 1990). The *Rho1*-induced phenotype was not observed with several other genes expressed from the pGMR promoter, including the the *Drosophila* protein kinases *cdc2*, *cdc2C* or *cdk5* or in

transgenic lines generated with the pGMR vector alone (J.de Nooij, L.-H.Tsai and I.K.Hariharan, unpublished observations).

To characterize the cellular abnormalities in the rough eyes, retinas from flies carrying 0, 1 or 2 copies (both as

Fig. 4. (A) A schematic representation of the *pGMR-Rho1* transposon (not drawn to scale). The *Rho1* cDNA was cloned into the *pGMR* plasmid (Hay *et al.*, 1994). In *pGMR*, the heat shock responsive elements in the promoter have been removed and replaced by five copies of a 29 bp binding site for the Glass protein. The Glass binding site targets expression of a reporter gene to all cells posterior to the morphogenetic furrow in the eye—imaginal disc (Ellis *et al.*, 1993). The 5' and 3' ends of the P element transposon are labelled 5' P and 3' P respectively. (B) A Northern blot. Total RNA from eye—antennal discs from wild-type and from *GMR-Rho1*³/*GMR-Rho1*³ larvae was electrophoresed, blotted and probed with a *Rho1* cDNA probe.

homozygous insertions and as two independent insertions) of the *Rho1* transgene were sectioned and examined microscopically (Figure 5E–H). Flies carrying two copies of the transgene in any configuration display a retinal phenotype characterized by a dramatic disruption of ommatidial architecture. The retina is markedly reduced in thickness. The lattice formed by the secondary and tertiary pigment cells is completely absent. Pigment cells are restricted to the apical regions of the retina and are likely to represent primary pigment cells. Photoreceptor cells of normal morphology are not evident, however, some rhabdomeres, often tortuous in morphology, are present below the layer of pigment cells (Figure 5H). Lens facets and ommatidial bristles are still present.

Flies carrying one copy of the transgene display a milder phenotype (Figure 5F). In contrast to the trapezoidal arrangement of rhabdomeres in wild-type ommatidia, the rhabdomeres are arranged in an irregular manner. The shape of the rhabdomeres also appears abnormal, some being irregular or bifid in cross-section. The number of photoreceptor cells in individual ommatidia appears to

be near normal in many cases, although the abnormal morphology of the cells and the rhabdomeres makes this difficult to define with precision.

Cell fate determination in the imaginal disc is unperturbed

The absence of normal ommatidia in flies carrying two copies of the *Rho1* transgene in a homozygous configuration could be due to a failure of cell proliferation or cell fate determination in the eye—imaginal disc. Alternatively, overexpression of *Rho1* may disrupt the differentiation of determined cells leading to their degeneration. To distinguish between these possibilities, imaginal discs were examined at various stages of development.

In normal eye—imaginal discs from third instar larvae, cells are progressively recruited into developing ommatidia. These developing ommatidia are regularly spaced and the rows are well organized. The nuclei of cells committed to a neuronal cell fate express the elav protein (Robinow and White, 1988). To determine whether recruitment of neurons to the eye disc is affected by *Rho1* overexpression, discs from wild-type and 'two copy' *rho* flies were stained with an elav-specific antibody (Figure 6A–F). In each cluster from wild-type discs, the developing photoreceptors stain in a characteristic sequence. R8, R2 and R5 are the first cells to express the antigen, followed by R3 and R4, then R1 and R6 and finally R7 (Figure 6E and F). Imaginal discs from 'two-copy' flies display a completely normal pattern of organization (Figure 6B, D, E and F). The ommatidia are regularly spaced and arranged in straight rows. Identical results were also obtained with the monoclonal antibody BP104 (Hortsch *et al.*, 1990), which stains a neuron-specific form of the neuroglian protein (data not shown). To determine whether cells in the cluster begin to differentiate as photoreceptor cells, larval imaginal discs were stained with the antibody 24B10, which recognizes the photoreceptor-specific antigen chaoptin (Zipursky *et al.*, 1984). The staining pattern observed in transgenic imaginal discs is indistinguishable from that observed in wild-type discs. Developing photoreceptor cells in the posterior part of the the imaginal disc stain and their axons extend through the posterior end of the disc down the optic stalk (Figure 6G).

Rho GTPases have been implicated in regulation of the actin cytoskeleton. Therefore, we examined the state of actin organization in developing retinas. Phalloidin stains filamentous actin and enables a visualization of the cell outlines as well as the structure of the actin cytoskeleton. Hence, eye—imaginal discs from late third instar larvae and from pupae at various stages were dissected, stained with phalloidin and examined using a confocal microscope. Retinal development appears to proceed relatively normally until at least 30 h after puparium formation (APF). By 40 h APF, in wild-type pupae the lattice of ommatidia is regular, the outlines of the cone cells, pigment cells and bristles are recognizable and the actin appears organized into neat bundles (Figure 7A and D). In the *GMR-Rho1*³ homozygotes the cytoskeleton appears extremely disorganized in all the ommatidia, although the outlines of the cone cells and pigment cells are still recognizable in many ommatidia (Figure 7B and E). Other ommatidia are in more advanced stages of degeneration (Figure 7C and F). These findings were also confirmed

Fig. 5. Effects of *pGMR-Rho1* on adult eyes. (A–D) Scanning electron micrographs (scale bar 100 μ m). (A) A wild-type eye; (B) a *GMR-Rho1*^{3/+} fly which carries one copy of the transgene; (C) a *GMR-Rho1*^{3/GMR-Rho1}³ fly which has two copies in a homozygous configuration; (D) a *GMR-Rho1*^{2/GMR-Rho1}³ fly which has two copies but which are located at two different genomic sites. (E and F) Sections of wild-type and *GMR-Rho1*^{3/+} retinas respectively (scale bar 10 μ m). (G) A section through a *GMR-Rho1*^{3/GMR-Rho1}³ retina (scale bar 100 μ m) which consists of a layer of pigment-containing cells and lacks the lattice formed by the secondary and tertiary pigment cells. (H) A higher magnification of a region of (G). Below the layer of pigment-containing cells there is no organized structure except for vesicles containing rhabdomere remnants (arrow) and clusters of rhabdomere-like structures (arrowheads).

by staining the retinas with cobalt sulfide (data not shown). At eclosion, the secondary and tertiary pigment cells are no longer visible in the retina and the remaining photoreceptors display grossly abnormal morphology (Figure 5G and H). Hence, the differentiation of both photoreceptor and pigment cells during the later stages of pupariation appears to be extremely disrupted.

Genetic modifiers of the *Rho1*-induced phenotype

Many of the components of the *Drosophila* ras pathway have been identified by dominant interactions between different mutations. For example, loss of one copy of any of several genes coding for downstream signalling components has been shown to attenuate ras-mediated signals from a temperature-sensitive (Simon *et al.*, 1991) or of a gain-of-function allele of *sevenless* (Olivier *et al.*, 1993), a transmembrane tyrosine kinase expressed in the R7 photoreceptor cell precursor. In light of the apparent connection between ras and rho signalling pathways in mammalian systems (Ridley *et al.*, 1992; Settleman *et al.*, 1992), we chose to examine the possibility that mutations of *Drosophila* ras pathway components might affect the severity of the *Rho1*-induced phenotype. In these experiments, flies carrying two copies of the transgene integrated on the same chromosome, *GMR-Rho1*¹ *GMR-Rho1*^{3/+}, were crossed with flies that are heterozygous for loss of function mutations in various components of the ras

pathway. These included mutations in the downstream receptor kinases (*drk*) adapter (Olivier *et al.*, 1993; Simon *et al.*, 1993), the *Son of sevenless* (*Sos*) gene, which encodes the ras guanine nucleotide exchanger (Simon *et al.*, 1991; Bonfini *et al.*, 1992), *Ras1* (Simon *et al.*, 1993), *Gap1*, which encodes the ras GTPase activating protein (Gaul *et al.*, 1992), *rolled* (*rl*), which encodes a MAP kinase homologue (Biggs *et al.*, 1994; Brunner *et al.*, 1994), *yan*, a putative nuclear target of this pathway (Lai and Rubin 1992) and *Rap1* (Hariharan *et al.*, 1991), a putative antagonist of the ras-mediated pathway (see Materials and methods). The intermediate nature of the *GMR-Rho1*¹ *GMR-Rho1*^{3/+} rough eye phenotype allows for the detection of either enhancement or suppression of eye roughness in the progeny of these crosses. However, examination of the derived flies under the microscope and histological analysis of their retinas failed to reveal any significant affect of any of these mutations on the *Rho1*-induced phenotype.

While mutations in the *Ras1* pathway failed to influence the phenotype, a preliminary survey of mutations in genes that have been implicated in signal transduction pathways indicated that four other mutations did so. A chromosome bearing an allele of *cdc37*, allele e1E (Cutforth and Rubin, 1994) (also known as *Enhancer of sevenless 3B*) suppresses the *Rho1* phenotype significantly. The sections of retinas of flies carrying two copies of the *GMR-Rho1* transgene

Fig. 6. Analysis of eye development in flies overexpressing *Rho1*. (A and C) Tissue from wild-type flies; (B, D, E and F) from *GMR-Rho1³/GMR-Rho1³* flies stained with an antibody directed against *elav*. (C) and (D) show regions from (A) and (B) at higher magnification. The arrowheads indicate the morphogenetic furrow. (E) A developing ommatidium just posterior to the furrow at an apical plane of focus at high magnification. The nuclei of photoreceptors 8, 2 and 5 are labelled. (F) A more posterior cluster at an apical focal plane. The nuclei of photoreceptors 3, 4, 1, 6 and 7 are indicated. (G) An imaginal disc from a *GMR-Rho1³/GMR-Rho1³* fly stained with an anti-chaoptin antibody 24B10 photographed at a basal focal plane. The arrowheads indicate axon bundles emerging from ommatidial clusters and forming an optic stalk (arrow).

lack recognizable ommatidia and the retina consists of a thin layer of pigment-containing cells (Figure 8A). When these flies are also heterozygous for the *cdc37^{el}* allele, secondary and tertiary pigment cells are once again observed to surround rhabdomeres of near normal morphology (Figure 8B) and a significant number of ommatidia are apparent (Figure 8C). The function of *cdc37* is unknown. *faint little ball* (*flb*) was found to be a weak suppressor and *tramtrack* (*ttk*) was a weak enhancer of the *Rho1*-induced phenotype (data not shown). *flb* encodes the *Drosophila* homologue of the EGF receptor (Schetjter and Shilo, 1989) and *ttk* encodes a transcription factor (Harrison and Travers, 1990). As would be expected for a *glass*-driven transgene, the phenotype is suppressed to some extent by a null allele of *glass* (Moses *et al.*, 1989) that should result in a reduced level of expression of the *Rho1* transgene. A number of other mutations tested show no significant effect (see Materials and methods). Currently, a more general mutational analysis is underway to identify genetic interactions with the observed *Rho1*-induced phenotype in the hope of identifying components of the *Rho1* signalling pathway. In a pilot screen, using ethyl methanesulphonate (EMS) as a mutagen, we have screened 8000 *F₁* progeny of mutagenized males and identified 14 enhancers and 9 suppressors, indicating that a relatively small number of loci can influence this

phenotype (K.Barrett, I.K.Hariharan and J.Settleman, unpublished observations).

Discussion

We have characterized three members of the rho GTPase family in *Drosophila*. The *Rho1*, *Rac1* and *Rac2* proteins are highly related structurally to their mammalian counterparts. Expression of each of these genes appears to be relatively widespread in *Drosophila*. This is also the case for mammalian *rac* and *rho* genes and is consistent with a requirement for the *rac* and *rho* GTPases in most if not all cell types. In germ band-extended embryos, the *Drosophila Rac1* gene is highly expressed in the mesoderm and in cells that give rise to the CNS. This might reflect an additional specialized function for *Rac1* in these tissues, as has been observed for mammalian *rac1* which regulates NADPH oxidase in human neutrophil leukocytes (Knaus *et al.*, 1991).

Much of our knowledge of *ras* function comes from studies of oncogenic *ras* alleles (Barbacid, 1987). Observations that mutationally activated *ras* or high levels of normal *ras* can induce proliferation or differentiation of cells in culture points to a role for *ras* in the regulation of these processes. Indeed, an activated *Drosophila Ras1* gene causes abnormalities in cell fate determination in

Fig. 7. Phalloidin staining of the pupal retina. Retinas from 40 h old pupae were stained with TRITC-conjugated phalloidin and visualized by confocal microscopy. (A and D) Tissue from a wild-type retina. (B, C, E and F) Tissue from *GMR-Rho1³/GMR-Rho1³* flies. Regions from (A–C) are shown at higher magnification in (D–F) respectively. (A) A cone cell is indicated by c and a primary pigment cell by p. (B) A region where the cell outlines are still recognizable. (C) A different region in the same retina which shows cells in a more advanced stage of degeneration. (D) The arrow points to a well-organized bundle of actin fibres in a wild-type retina. (E and F) The arrows point to the disorganized actin structures. The scale bar (in F) represents 9 μ m for panels A–C and 5 μ m for panels D–F.

the larval imaginal disc leading to the specification of additional R7 photoreceptor cells (Fortini *et al.*, 1992). In contrast, overexpression of *Rho1* in cells of the developing eye does not appear to affect cell fate determination in the eye–imaginal disc, despite causing a dramatic rough eye phenotype. In imaginal discs stained with a variety of antibodies, cells are recruited to a neuronal fate in a normal sequence.

In flies overexpressing *Rho1*, many photoreceptor cells, as well as almost all secondary and tertiary pigment cells seem to degenerate during the late pupal phase of development. In the classification of eye development mutants of Renfranz and Benzer (1989), the *GMR-Rho1* homozygotes would be classified in Group IV, which includes mutations which affect terminal differentiation. This is later than the block in differentiation observed in *glass* mutants, in which photoreceptor-specific antigens such as chaoptin are not expressed (Moses *et al.*, 1989), and earlier than the defect observed in *disconnected*, where

photoreceptor cell death begins several days after eclosion due to a failure to form connections with the optic lobes (Steller *et al.*, 1987).

Abnormal retinal development in *GMR-Rho1* homozygotes is coincident with a phase when the morphology of the photoreceptor and the secondary and tertiary cells undergo dramatic changes. These cells increase in length as the entire retina increases in thickness. Cells that do not span the thickness of the retina, such as the primary pigment cells, seem to be less vulnerable to the deleterious effects of the overexpression of *Rho1*. Since activation of *rho* induces actin filament assembly, an attractive notion is that increased *Rho1* expression at this stage impedes the ability of the cytoskeleton to tolerate marked changes in shape. The lack of an obvious *Rho1*-induced phenotype in the larval imaginal disc is not due to lack of expression of the transgene at this stage, as we have been able to detect its expression in the eye–imaginal discs of third instar larvae by Northern blotting and by *in situ*

Fig. 8. Suppression of the *Rho1*-induced phenotype. (A) A longitudinal section through a retina from a *GMR-Rho1¹ GMR-Rho1^{3/+}* fly (scale bar 25 μ m). The retina is thin and contains a thin layer of pigment-containing cells (arrowhead). Below this layer the retina lacks recognizable structure (arrow). (B and C) Sections from flies which are *GMR-Rho1¹ GMR-Rho1^{3/cdc37^{elE}}*. In these flies the retinal thickness has increased (B) and the pigment cells (arrowed) extend along and surround the rhabdomeres of the photoreceptor cells. Recognizable ommatidia are once again apparent (C).

hybridization. Furthermore, other transgenes using this vector are well expressed at early stages of development (our unpublished results; Ellis *et al.*, 1993). It is possible, however, that even higher levels of *Rho1* transgene expression may result in deleterious effects at earlier stages in development, since changes in cell shape occur even in the larval imaginal disc.

The normal role of the *Rho1* gene in eye development remains to be established. Expression of *Rho1* in the embryo and in the imaginal discs (data not shown) appears to be widespread. The ubiquitous expression of *Rho1* argues for a function in most cells. We attempted to disrupt the normal functioning of *Rho1* in the eye—imaginal disc by establishing transgenic flies harbouring a dominant-negative version of the *Rho1* gene. However, despite numerous injection efforts, we were unable to recover transgenic flies with dominant-negative versions of the *Rho1* cDNA. We suspect that the artificial pGMR promoter is leaky, resulting in a low level of widespread expression. This could result in lethality during development for flies bearing a dominant-negative *Rho1* transgene. The specific requirements for the various rho family GTPases in development might eventually be elucidated by the identification of loss-of-function mutations in these genes. The cloning and mapping of the *rho* and *rac* homologues reported here should facilitate the identification of such mutations.

Since the phenotype in the eye was induced by over-expressing a wild-type form of *Rho1*, it is likely that it can be influenced by levels of proteins that function either upstream or downstream of *Rho1*. Ridley *et al.* (1992) demonstrated that *ras* can activate *rho* and *rac* in fibroblasts. However, they also demonstrated that interfering with the function of *ras* does not block the ability of a variety of extracellular growth factors to activate *rac* and *rho*. In our experiments as well, heterozygous loss of function mutations in *ras* pathway components do not appear to influence the *Rho1* phenotype. However, it is possible that the *ras* and *rho* pathways interact in a way that is not revealed in this assay or, alternatively, that such interactions are restricted to other cell types. We have, however,

shown that several other mutants can influence the *Rho1*-induced phenotype.

The mechanisms that regulate cell shape changes during the development of an organism are poorly understood. While numerous mutations have been identified in *Drosophila* that affect cytoskeletal organization, it remains unclear how the products of such genes regulate the precise morphologic changes that accompany specific developmental events. Exhaustive genetic screens for mutants that disrupt gastrulation in the embryo have, so far, identified two genes that might specifically be involved in regulating the apical constriction of cells in the ventral furrow, *concertina* (Parks and Wieschaus, 1991) and *folded gastrulation* (Costa *et al.*, 1994). It has been postulated that additional loci have not been identified because those proteins are supplied both maternally and zygotically (Costa *et al.*, 1993). Based on the apparent role of the rho family GTPases in fibroblasts, it seems very likely that they are involved in the regulation of cell morphology in response to extracellular signals. The eventual identification of rho pathway components may reveal the mechanism by which the rho GTPases mediate such morphological changes.

Materials and methods

Cloning of *rho* and *rac* homologues

Pairs of degenerate synthetic oligonucleotides (containing *EcoRI* and *BamHI* sites) were mixed with 1 μ g of *Drosophila* genomic DNA in a buffer containing nucleotides and *Taq* polymerase and subjected to thermal cycling. The first five cycles were performed under low stringency annealing conditions (94°C 1 min, 37°C 1 min, 72°C 1 min). The next 30 cycles were performed at higher stringency (94°C 1 min, 50°C 1 min, 72°C, 2 min). Amplified DNA products were digested with *BamHI* and *EcoRI*, purified following gel electrophoresis and subcloned into pBluescript (Stratagene) for sequence analysis using standard methodology.

A *Drosophila* imaginal disc cDNA phage library (5×10^5 clones) (Novagen) was screened in duplicate under slightly relaxed stringency conditions. Boiled 32 P-labelled probe (1 ng/ml) was added to a solution of $5 \times$ SSC, $2 \times$ Denhardt's, 40% formamide, 0.1% SDS and 100 μ g/ml salmon sperm DNA and hybridization was performed for 16 h at 42°C. Clones which remained positive upon secondary and tertiary screening were subjected to plasmid rescue from the phage. cDNA inserts were subcloned into pBluescript for sequence analysis. To facilitate sequence analysis, nested deletions of the cloned inserts were created using Erase-a-base (Promega). DNA sequencing was performed with Sequenase (US Biochemical).

RNA in situ hybridizations

Digoxigenin-labelled RNA probe synthesis was performed according to Boehringer Mannheim protocols. *Rac1*, *Rac2* and *Rho1* cDNAs were each cloned into the vector pSELECT. SP6 polymerase was used to synthesize antisense RNAs of *Rac1* and *Rac2* after the inserts were linearized with *EcoRI*. T7 was used to make the corresponding sense strands following linearization of the inserts by *HindIII*. In the case of *Rho1*, which has been cloned in both directions, the plasmids were linearized using *BamHI* and T7 was used to make both the antisense and sense strand probes. *In situ* hybridization to whole-mount embryos was performed using digoxigenin-labelled RNA probes (Boehringer Mannheim), as described by Tautz and Pfeiffle (1989), except that 4 μ g/ml of proteinase K was used.

Construction of *Rho1* transgenic lines

A 779 bp *EcoRI* fragment containing the entire coding region fragment of the *Rho1* cDNA was cloned into the pGMR vector (Hay *et al.*, 1994). The pGMR-*Rho1* plasmid DNA was co-injected with the pUCHs Δ 2-3 helper plasmid into *w¹¹¹⁸* embryos. Transformation of *D.melanogaster* was performed as described in Spradling and Rubin (1982) and Rubin and Spradling (1982).

Northern blot analysis

Northern analysis was exactly as described by Fischer-Vize *et al.* (1992), except that RNA from 10 pairs of eye-antennal discs was loaded in each track.

Scanning electron microscopy

Samples for SEM were prepared as described by Kimmel *et al.* (1990), except that critical point drying was used to dehydrate adult *Drosophila*.

Immunohistochemistry

Heads from adult flies were embedded in Durcupan resin and 2 µm sections were cut on a Reichert Jung microtome essentially as described by Tomlinson and Ready (1987a) with the modifications described by Carthew and Rubin (1990). The sections were stained in a solution of toluidine blue (1%) and borax (1%), mounted in DPX (Fluka) and viewed under bright field illumination.

Eye-imaginal discs were stained with antibodies as described by Tomlinson and Ready (1987b), although for the anti-elav antibody, 0.1% Triton X-100 was used instead of saponin and the intensification was with cobalt chloride (1.5 mM) and nickel chloride (2.5 mM) instead of osmium tetroxide. The anti-elav antibody and the 24B10 antibodies were used at a 1:2 dilution.

White pre-pupae were cultured at 25°C. For phalloidin staining, pupal retinas were dissected in phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde in PBS for 15 min at room temperature, washed for 15 min in PBS containing 0.3% Triton X-100 and incubated overnight at 4°C in PBS containing 5% (v/v) normal goat serum and 5% (v/v) of a stock solution of phalloidin. The stock solution was prepared by dissolving 0.1 mg TRITC-labelled phalloidin (Sigma) in 4 ml of 100% ethanol. Following the overnight incubation, the retinas were rinsed in PBS, mounted in a glycerol mounting solution (90% glycerol, 1× PBS, 0.5% *n*-propyl gallate) and viewed using a Bio-Rad MRC-600 confocal system attached to a Zeiss Axiocvert compound microscope. Cobalt sulfide staining of pupal discs was performed as described by Cagan and Ready (1989).

Fly crosses

All fly crosses were performed at 25°C. The alleles used were: *drk* (*e0A*), *Sos* (*e4G*, *e6C*), *Ras1* (*e1B*, *e2F*) (Simon *et al.*, 1991), *rl* (*X16z*, *S-694*) (from Tyler Cutforth), *Rap1* [*rv(R)*^{B1} and *rv(R)*^{B3}] (Hariharan *et al.*, 1991), *cdc37* (*e1E*) (Cutforth and Rubin, 1994), *fib* (*IK-35*), *glass* (*60j*) (Moses *et al.*, 1989) and *ttk* [*P(ry)5125* and *P(ry)3540*] (from the Spradling laboratory; Karpen and Spradling, 1992). Other mutations tested which displayed no interaction with the *Rho1*-induced phenotype included alleles of *hedgehog*, *sina*, *zerknult*, *screw*, *fat facets*, *twisted gastrulation*, *shrew*, *cactus*, *spitz* and *DC0*, which encodes the catalytic subunit of cAMP dependent kinase (Lindsley and Zimm, 1992). In the screen for modifiers of the *Rho1*-induced phenotype, *w¹¹¹⁸*, *iso2*, *iso3* males were fed with a solution containing 25 mM EMS and crossed to *GMR-Rho1¹GMR-Rho1³TM6B* *Hu* females. The F₁ progeny that were *Hu*⁺ were screened.

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