Ras controls growth, survival and differentiation in the *Drosophila* eye by different thresholds of MAP kinase activity

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SUMMARY

Ras mediates a plethora of cellular functions during development. In the developing eye of *Drosophila*, Ras performs three temporally separate functions. In dividing cells, it is required for growth but is not essential for cell cycle progression. In postmitotic cells, it promotes survival and subsequent differentiation of ommatidial cells. In the present paper, we have analyzed the different roles of Ras during eye development by using molecularly defined complete and partial loss-of-function mutations of *Ras*. We show that the three different functions of Ras are mediated by distinct thresholds of MAPK activity. Low MAPK activity prolongs cell survival and permits differentiation of R8 photoreceptor cells while high or persistent MAPK activity is sufficient to precociously induce R1-R7 photoreceptor differentiation in dividing cells.

Key words: Ras1, Signal transduction, Cell growth, Cell death, Differentiation, *Drosophila*

INTRODUCTION

Activation of the small GTPase Ras is associated with a wide variety of cellular responses to extracellular stimuli (Campbell et al., 1998; Rommel and Hafen, 1998). In cultured mammalian cells, Ras is activated in response to mitogenic signals, and interfering with Ras activation causes growth arrest. Oncogenic transformation of cells is frequently associated with the constitutive activation of Ras. In addition to proliferation, Ras activity is also involved in differentiation. In *Caenorhabditis elegans*, Ras controls larval viability, vulva induction and male spicules development (Sternberg and Han, 1998). In *Drosophila*, Ras activity has multiple functions including the specification of larval head and tail structures, mediating signaling by the Torso receptor tyrosine kinase, specification of ventral ectoderm fate in the embryo and dorsoventral polarity in the egg shell in response to EGF receptor stimulation. In imaginal discs, Ras is required for cell growth, differentiation of wing veins and photoreceptor cells (Diaz-Benjumea and Hafen, 1994; Freeman, 1998; Prober and Edgar, 2000). Overexpression of constitutively active Ras induces tissue overgrowth and non-autonomous cell death (Karim and Rubin, 1998).

Three different models have been proposed to explain how Ras can control different cellular responses. In the first model, the cellular response to Ras activity is dictated by the cellular context. For example, Ras signaling regulates different sets of transcription factors in response to epidermal growth factor (EGF) receptor and Sevenless activation in the developing eye, and in response to Torso activation in the embryo (reviewed in Freeman, 1998; Dickson, 1995; Lu et al., 1993). In *C. elegans*,

specification of tail or vulval structures in response to Ras activity depends on the expression of different homeodomain proteins (Maloof and Kenyon, 1998). When we take this model to the extreme, Ras activation is a mere trigger for a single cellular response. In the second model, the combined activities of different signal transduction pathways determine the biological response. For example, transformation of fibroblasts in response to oncogenic Ras depends on the ability of constitutively active Ras to activate two distinct effectors, Raf and PI3K (Rodriguez-Viciana et al., 1997). In the third model, quantitatively different levels of Ras activity elicit qualitatively different responses. For example, stimulation of PC12 cells by EGF induces Ras and MAP kinase activity transiently and induces proliferation. Stimulation by NGF, however, results in a prolonged activation of Ras and MAP kinase and triggers neurite outgrowth (Marshall, 1995). In the *Drosophila* embryo, different amounts of constitutively active Ras activate different target genes and cause the differentiation of different posterior structures (Greenwood and Struhl, 1997; Ghiglione et al., 1999).

One problem concerning the experimental evidence that underlies each of the three different models is the fact that most experiments involve the overexpression of constitutively active Ras protein and hence may not reflect a physiological situation. We have analyzed Ras function in the developing eye in vivo using molecularly defined *Ras* mutations that affect a specific signaling pathway. In the developing eye, complete loss of Ras function slows down cell growth but cells can still progress through the cell cycle. However, *Ras* null mutant cells fail to differentiate and undergo apoptosis upon exit from the cell cycle. Ras^{D38E}, a Ras effector mutant with reduced activity

towards Raf, rescues survival of postmitotic cells and differentiation of the R8 photoreceptor cells in the eye disc but fails to promote recruitment of the other photoreceptors. Differentiation of R1-R7 cells can be rescued, however, by a concomitant increase in mitogen-activated protein kinase (MAPK) activity. Furthermore, high levels of Ras activity in proliferating cells in the eye imaginal disc result in precocious neuronal differentiation. These results indicate that different thresholds of Ras activity specify different cellular responses and that all these responses are mediated by the Raf/MAPK effector pathway of Ras.

MATERIALS AND METHODS

Fly stocks

Ras^{x7b} is an excision allele of the P-element line Ras^{5703} (Schnorr and Berg, 1996). $PI3K$ alleles used are $Dp110^A$ (Weinkove et al., 1999) and $Dp110^{2H1}$, which was isolated in a screen for growth defective mutations (H. S. and E. H., unpublished). Flies carrying P(sev-Ras^{G12V},w^+), P(sev-$Ras^{G12V,T35S}$,w^+), P(sev-$Ras^{G12V,D38E}$,w^+), P(sev-$Ras^{G12V,Y40C}$,w^+); P(UAS-Ras^{G12V},w^+), P(UAS-$Ras^{G12V,D38E}$,w^+), P(UAS-$Ras^{G12V,Y40C}$,w^+), P(UAS-Raf^{torY9},w^+); and P(Ras,y^+), P(Ras^{D38E},y^+), P(Ras^{Y40C},y^+) were generated by P-element transformation. For the overexpression studies, we used the lines sev-Ras^{G12V}-1 (2nd chr.), sev-$Ras^{G12V,T35S}$-2 (3rd chr.), sev-$Ras^{G12V,D38E}$-2 (3rd chr.), sev-$Ras^{G12V,Y40C}$-1 (3rd chr.) and UAS-Ras^{G12V}-2 (3rd chr.), UAS-$Ras^{G12V,D38E}$-3 (2nd chr.), UAS-$Ras^{G12V,Y40C}$-2 (2nd chr.), UAS-Raf^{torY9}-62 (2nd chr.). For clonal analyses we used transformants of P(Ras): Ras-3 (2nd chr.), Ras^{D38E}-27 (2nd chr.), Ras^{D38E}-29 (2nd chr.), Ras^{Y40C}-23 and Ras^{Y40C}-30 (2nd chr.). Line $Ras^{D38ExD38E}$-13 contains the two recombined P elements of the lines Ras^{D38E}-27 and Ras^{D38E}-29. Lines $Ras^{Y40CxY40C}$-7 and $Ras^{Y40CxY40C}$-15 arose from two independent recombination events of the P elements of line Ras^{Y40C}-23 and line Ras^{Y40C}-30. Lines $Ras^{D38ExY40C}$-8 and $Ras^{D38ExY40C}$-14 arose from two independent recombinations of the P elements of line Ras^{D38E}-27 and line Ras^{Y40C}-23. The recombination events were confirmed by Southern analysis.

Molecular analysis of Ras^{x7b}

The 1.7 kb deletion of Ras^{x7b} starts 18 bp upstream of the transcription start and ends 198 bp downstream of the putative polyadenylation signal. The deletion extent was determined by PCR using the following flanking primers: oth02, GAGCCTCTTTTGTCTTTCGC; oth06, CAGTGCGTTCTATTTTGGCC; oth11, GCTGTATTGAT-CAAATCATTTATT; and RasHind, GGCTGTTTGTTTTCTCTCCG.

Plasmid construction

A 3.6 kb HindIII/HindIII fragment containing the *Drosophila Ras1* gene (cloned out of a cosmid library) was cloned into a BlueScript vector in which the SalI and XbaI sites had been deleted. Since the HindIII/HindIII fragment also contains the first 800 bp of the *Rlb1* gene we exchanged the SalI/HindIII fragment of the *Rlb1* gene with a fragment containing a deletion of one nucleotide at position 12 which results in a frameshift and a predicted peptide of three amino acids (ΔRlb1; Schnorr and Berg, 1996). In addition, mutations in the Ras effector domain (D38E, Y40C) were generated by PCR-mediated in vitro mutagenesis on a SalI/EcoRI fragment of *Ras* cloned into the BlueScript vector and confirmed by sequencing. The different mutations were introduced into the BS-ΔRlb1Ras1$^+$ vector as a SalI/XbaI fragment. The ΔRlb1Ras1$^+$ insert was excised as a Asp 718/NotI fragment and cloned into a modified y$^+$-marked Carnegie vector (pTH4). For the overexpression studies we generated the *Ras* effector mutants (T35S, D38E, Y40C) by PCR-mediated in vitro mutagenesis on the cDNA of Ras^{G12V}. The mutant *Ras* genes were then cloned into the 2xsevEsevP vector and into pUAST vector. P-

element-mediated transformation was carried out as described (Rubin and Spradling, 1982).

Clonal analysis

Mitotic recombination was induced using the FLP/FRT technique (Xu and Rubin, 1993). $Ras^{-/-}$ clones in the eye imaginal disc were induced by heat shock induction of FLP recombinase in y w $hsFLP;P(Ras,y^+)/+;FRT82B$ $Ras^{x7b}/FRT82B$ $P(arm\text{-}lacZ)$ larvae 24-48 hours AED for one hour at 37°C. Clones in a *Minute* background were induced in y w $hsFLP;P(Ras,y^+)/+;FRT82B$ $Ras^{x7b}/FRT82B$ $P(arm\text{-}lacZ)$ $M(3)w^{124}$ larvae 24-48 hours AED for 30 minutes at 37°C.

To determine the clonal size in wing discs, clones were induced by heat shock in y w $hsFLP;P(Ras,y^+)/+;FRT82B$ $Ras^{x7b}/FRT82B$ $P(arm\text{-}lacZ)$ larvae 48 hours AED (4 hours egg collections) for 10 minutes at 34°C and analyzed at 120 hours AED.

For generating $Ras^{-/-}$ clones in the adult eye, either larvae of the genotype y w $hsFLP;P(Ras,y^+)/+;FRT82B$ $Ras^{x7b}/FRT82B$ w^+ were heat shocked 24-48 hours AED for one hour at 37°C to induce mitotic recombination or we selectively removed Ras function in the eye by using $eyFLP;FRT82B$ $Ras^{x7b}/FRT82B$ w^+ $cl3R3$ (Newsome et al., 2000), where a cell lethal mutation is combined with the Ras^+ chromosome and therefore the only proliferating cells in the eye imaginal disc are homozygous mutant for *Ras*. Adults were examined for w clones in the eye. Histological sections of the eyes were performed as described previously (Basler and Hafen, 1988).

Gain-of-function clones of Ras were induced using the FLP-out technique (Struhl and Basler, 1993; Neufeld et al., 1998). $hsFLP;$ $Act5c>CD2>Gal4,UAS\text{-}GFPnls$ $+/-$ additional UAS lines larvae were heat shocked at 48-72 hours AED for 30 minutes at 34°C.

Discs were fixed for 20 minutes in 4% paraformaldehyde in PBS and then permeabilized with 0.3% Triton X-100 in PBS. Following antibodies were used: rat anti-Elav (1:30, a gift from G. Rubin), rabbit anti-β-galactosidase (1:2000, Cappel), rabbit anti-Boss (1:2000, a gift from L. Zipursky). Texas Red- and FITC-conjugated secondary antibodies (1:200, Jackson ImmunoResearch) were used.

TUNEL assay

Apoptotic cells were detected using the ApopTaq system (ONCOR). The 3′-OH ends of DNA were labeled for one hour at 37°C by addition of digoxigenin 11-UTPs by the enzyme TdT and subsequently detected with a FITC-conjugated anti-digoxigenin antibody.

RESULTS

A set of molecularly defined partial and complete loss-of-function mutations in *Ras*

Ras function in the developing eye has been studied extensively by overexpression of constitutively active forms of Ras and by reducing Ras function in heterozygous conditions (reviewed by Wassarman et al., 1995). These studies have enabled us to establish a crucial role for Ras in the differentiation of photoreceptor cells. Little is known, however, about earlier functions of Ras in eye development, as complete loss of Ras function causes larval lethality. The use of clonal analysis of mutant *Ras* alleles of different strength has been useful in dissecting Ras function in the adult wing (Diaz-Benjumea and Hafen, 1994). We wanted to use an allelic series of molecularly defined *Ras* mutations to dissect the different roles of Ras during eye development and to address the question of whether quantitatively different levels of Ras and its downstream effector Raf define thresholds for different cellular responses. We first characterized a precise deletion mutation in *Ras*

(Ras^{x7b}) that serves as a complete loss-of-function mutation. Furthermore, we generated a genomic *Ras* transgene that rescues the lethality associated with the *Ras* null allele, and thus appears to contain all regulatory sequences necessary for the correct spatial and temporal expression of *Ras*. By introducing mutations into this rescue construct we can analyze the effects of partial loss-of-function mutations under physiological conditions rather than rely on overexpression or constitutive activation.

In mammalian cells, single amino acid substitutions in the effector domain of Ras in conjunction with the constitutively active G12V mutation have been used successfully to dissect the role of different Ras effectors in cellular responses (White et al., 1995; Joneson et al., 1996). Expression of the constitutively active Ras^{G12V} in mammalian cells activates, in addition to Raf other signaling components such as PI3K (Rodriguez-Viciana et al., 1997). In these cells, $Ras^{G12V,D38E}$ or $Ras^{G12V,T35S}$ exhibit a reduced activity towards Raf and fail to detectably activate PI3K. Conversely, $Ras^{G12V,Y40C}$ exhibits reduced activity towards PI3K but has no detectable activity towards Raf. Given the identity of the amino acid sequence between mammalian H-Ras and *Drosophila* Ras in and around the effector domain, we hypothesized that these mutations would have a similar effect on the activity of *Drosophila* Ras. To test the specificity of these mutant proteins in *Drosophila*, we investigated their ability to induce phenotypes characteristic for the activation of the putative downstream effectors Raf and PI3K, respectively. Differentiation of extra R7 photoreceptor cells is induced by expression of constitutively active components of the Ras/MAP kinase pathway in the progenitors of the R7 and the lens-secreting cone cells using the enhancer of the *sevenless* (*sev*) gene. Flies carrying a *sev-Ras^{G12V}* transgene possess rough eyes, owing to the presence of additional R7 photoreceptor cells (Fig. 1B, arrow; Fortini et al., 1992). Flies carrying one copy of the *sev-$Ras^{G12V,D38E}$* transgene also showed a characteristic multiple R7 phenotype (Fig. 1C, arrow). We note, however, that the phenotype is considerably weaker than that observed in *sev-Ras^{G12V}* flies. Identical results were obtained with the $Ras^{G12V,T35S}$ transgene (data not shown). Flies carrying one copy of the $Ras^{G12V,Y40C}$ transgene had wild-type eyes with no extra R7 cells (Fig. 1D). Thus, in the Ras effector domain, the D38E and the T35S substitution are able to support neuronal development in the context of an activating G12V substitution, while the Y40C substitution is not. These results indicate that, albeit at a reduced level, $Ras^{G12V,D38E}$ and $Ras^{G12V,T35S}$ are able to activate Raf, while $Ras^{G12V,Y40C}$ is not.

Overexpression of PI3K in postmitotic cells under the control of *GMR-Gal4* increases the size of the eye but does not affect eye patterning (Leevers et al., 1996). In contrast, *GMR-Gal4, UAS-$Ras^{G12V,Y40C}$* flies had rough eyes but showed no significant increase in eye size (Fig. 1E). Expression of Ras^{G12V} and $Ras^{G12V,D38E}$ under *GMR-Gal4* control caused lethality, most likely due to the high Ras activity towards Raf. The rough eye phenotype in *GMR-Gal4, UAS-$Ras^{G12V,Y40C}$* flies was not caused by a hyper-activation of PI3K, as concomitant reduction of endogenous PI3K function caused a reduction in head and body size but did not suppress the rough eye phenotype (Fig. 1F). Based on this genetic evidence, it appears that $Ras^{G12V,Y40C}$ is not able to trigger PI3K-specific cellular responses in the developing
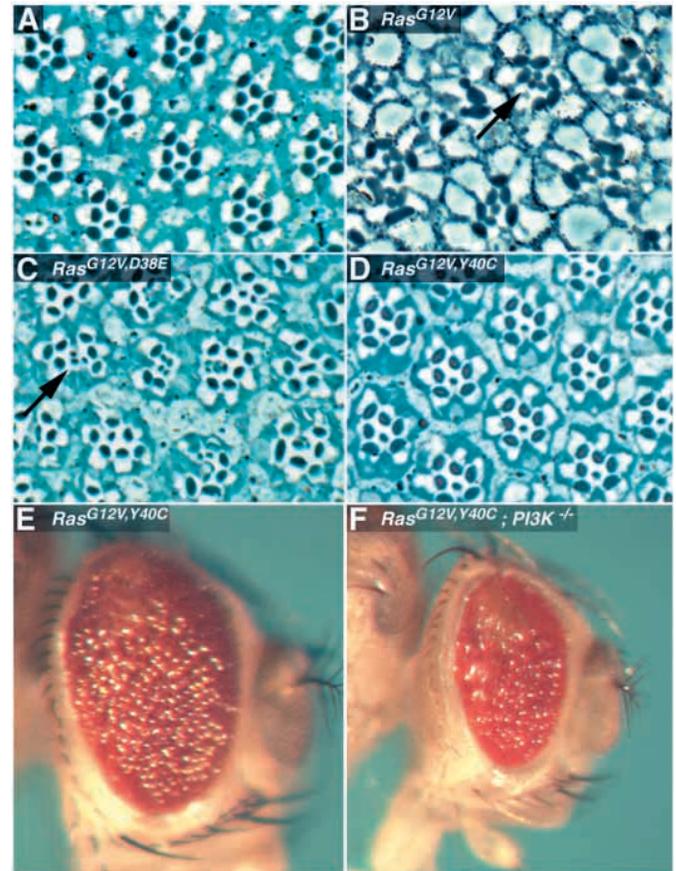

Fig. 1. In the context of a constitutively activated Ras protein, Ras^{D38E} is sufficient to induce R7 differentiation whereas the Ras^{Y40C}-induced rough eye phenotype is not PI3K-dependent. (A-D) Tangential sections of compound eyes of wild-type (A), *sev-Ras^{G12V}* (B), *sev-$Ras^{G12V,D38E}$* (C) and *sev-$Ras^{G12V,Y40C}$* (D) flies. Additional R7 cells are recruited in each ommatidium in Ras^{G12V} eyes (B, arrow) and in $Ras^{G12V,D38E}$ eyes (C, arrow). There is no recruitment of additional R7 cells in $Ras^{G12V,Y40C}$ eyes (D). (E-F) Head of a *GMR-Gal4/UAS-$Ras^{G12V,Y40C}$* fly (E) and a *GMR-Gal4/UAS-$Ras^{G12V,Y40C}$; $Dp110^{A}/Dp110^{2H1}$* fly (F). The $Ras^{G12V,Y40C}$ transgene causes a rough eye phenotype (E). Reducing PI3K function by a viable combination of *Dp110* loss-of-function alleles results in proportionally smaller flies with smaller eyes (F) but no suppression of the rough eye phenotype.

eye. $Ras^{G12V,Y40C}$ may thus interfere with or activate another yet unknown pathway leading to the formation of rough eyes. Several studies in *Drosophila* have used the Ras effector site mutations to distinguish between possible downstream effectors in the control of specific cellular responses (Bergmann et al., 1998; Therrien et al., 1999). Our analysis indicates that in spite of the conservation of the Ras effector domain these mutations may not have similar effects: while Ras^{D38E} and Ras^{T35S} are able to activate Raf, Ras^{Y40C} appears unable to activate Dp110 PI3K.

Ras function is required for growth, survival and neural differentiation

In order to test the role of Ras in the development of ommatidial cells we generated clones of cells homozygous for the null mutation Ras^{x7b}. $Ras^{-/-}$ clones could not be recovered

Fig. 2. Ras function is required for growth, survival and differentiation. (A-B) Analysis of $Ras^{-/-}$ clones in the adult eye. Mitotic clones of Ras^{x7b} were induced 24-48 hours after egg deposition (AED) by heat shock. Mutant clones are marked by the lack of pigment. $Ras^{-/-}$ photoreceptors are not detected in the adult eye, but the regular ommatidial pattern is disrupted (A). $Ras^{-/-}$ photoreceptors are rescued by the Ras rescue construct (B). (C-G) Analysis of $Ras^{-/-}$ clones in third instar eye imaginal discs. Mitotic clones of Ras^{x7b} were induced 24-48 hours AED by heat shock. Discs were double labeled with an antibody to β-galactosidase (green, left panel) and in C,D,F,G, with a second antibody to the nuclear neuronal marker Elav (red, center panel). (E) TUNEL-positive apoptotic cells (red, center panel) were detected by using a FITC-labeled anti-digoxigenin antibody upon incorporation of digoxigenin nucleotides to the 3′-OH ends of DNA. The merged images are shown in the right-hand panel. Mutant clones are marked by the absence of anti-β-galactosidase staining, the corresponding twin spots by two copies of *lacZ* resulting in a more intense staining (C,E,F). Posterior is to the bottom. Cells lacking Ras function proliferate but do not express Elav within the morphogenetic furrow and disappear behind the morphogenetic furrow (C). $Minute^{+}Ras^{-}$ clones in a *Minute* background cover large areas of the discs (D). Clusters of TUNEL-positive apoptotic cells were detected in $Ras^{-/-}$ clones only posterior to the morphogenetic furrow (E). All Ras phenotypes in the eye imaginal disc are rescued by a single copy of the Ras rescue construct in a wild-type background (F) and in a *Minute* background (G).
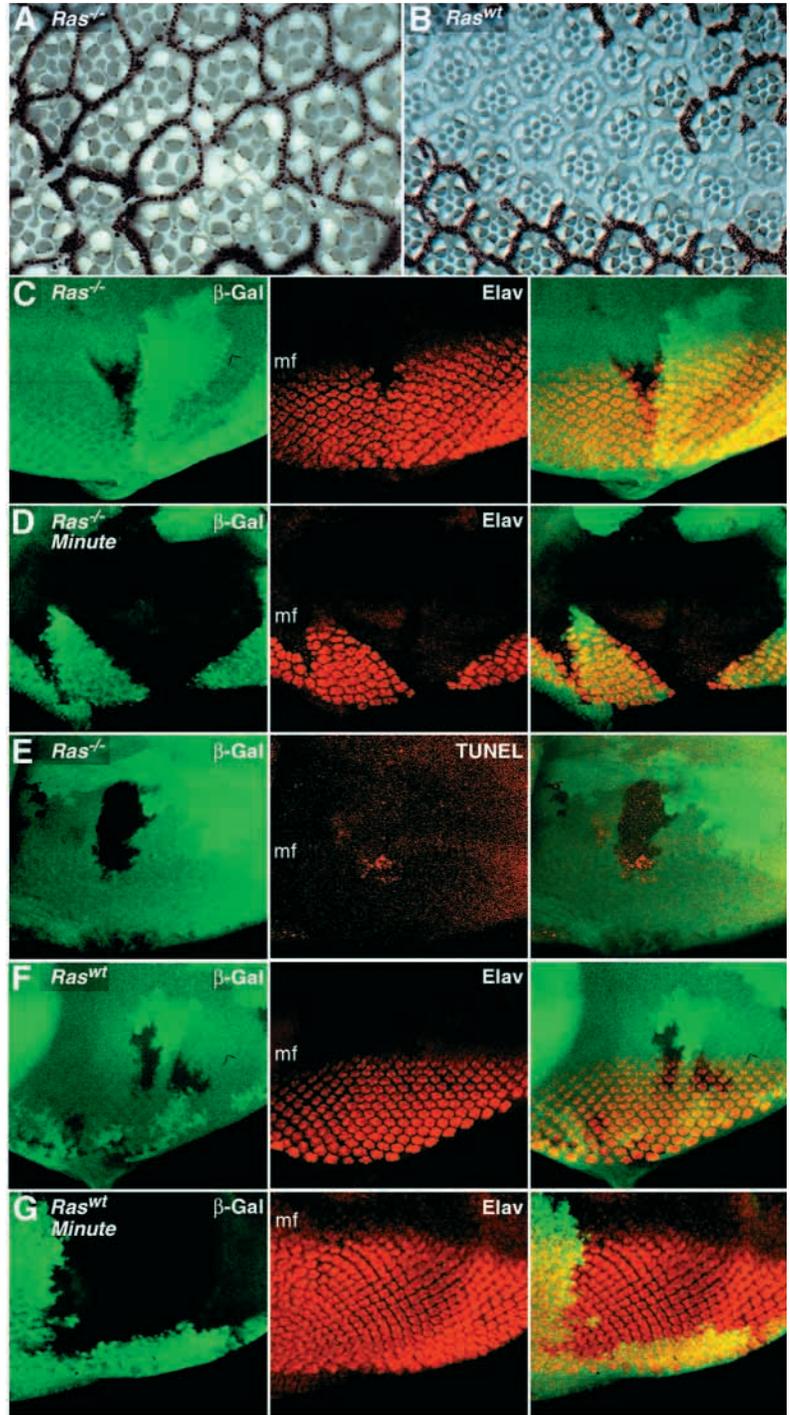
in the adult eye, suggesting that Ras is essential for proliferation, growth or survival of eye imaginal disc cells (Simon et al., 1991; Fig. 2A). When we examined *Ras* mutant clones in eye imaginal discs we made two observations (Fig. 2C). First, clones of $Ras^{-/-}$ cells are observed in the eye imaginal disc; however, compared with that of their wild-type sister clones (twin spots), the relative size of the clones is reduced (which indicates that cells lacking Ras function can proliferate but that they have a growth disadvantage). Second, *Ras* mutant clones located behind the morphogenetic furrow are often wedge-shaped and much reduced in size. It appears that $Ras^{-/-}$ cells disappear once the morphogenetic furrow has passed. Behind the morphogenetic furrow, cells stop dividing, are recruited into ommatidial clusters and differentiate into photoreceptors and non-neuronal cone or pigment cells (Wolff and Ready, 1991). Thus, it appears that Ras function is not essential for proliferation, whereas it is essential for survival and/or differentiation of postmitotic cells in the eye disc.

The growth disadvantage of $Ras^{-/-}$ cells is not an intrinsic defect in the *Ras* mutant cell but it is caused by a failure to compete successfully with faster growing $Ras^{+/-}$ cells. If the growth rate of the heterozygous $Ras^{+/-}$ cells is reduced by a dominant *Minute* (*M*) mutation, the $Ras^{-/-}$ clones are substantially larger and cover large areas of the disc (Fig. 2D). Similar results were also obtained in the wing disc (data not shown). As observed in a wild-type background, the size of

$Ras^{-/-}$ clones in the *M* background is reduced behind the morphogenetic furrow, suggesting that *Ras* mutant cells fail to differentiate and are eliminated autonomously. Indeed, we observed a significant increase in apoptotic cells within $Ras^{-/-}$ clones when we performed TUNEL staining on imaginal discs (Fig. 2E).

The analysis of *Ras* loss-of-function clones has enabled us to distinguish three temporally separate functions of Ras in the developing eye imaginal disc. First, Ras function contributes to normal cell growth. *Ras* mutant cells have a growth disadvantage and cannot compete with wild-type cells, but it

appears, however, that Ras function is not essential for cell cycle progression. Second, Ras function is required for the survival of postmitotic ommatidial cells. Third, Ras function is necessary for neuronal differentiation of photoreceptor cells. All the three phenotypes are rescued by a single copy of the *Ras* transgene (Fig. 2B, 2F and 2G).

Partial rescue of *Ras* mutant phenotypes by effector site mutants

To test whether Ras variants containing amino acid substitutions in the effector domain can rescue any of these three distinct requirements for Ras during eye development we introduced the D38E and the Y40C mutations in the genomic *Ras* rescue construct. We generated transgenic lines that contained one or more copies of the different mutant constructs in combination with the *Ras* null allele. While one copy of the wild-type rescue construct rescued the lethality of homozygous *Ras* mutant animals, the presence of one or two copies of any of the mutant transgenes did not rescue *Ras* mutants to adulthood. Similarly, none of the mutant constructs was sufficient to rescue $Ras^{-/-}$ clones in the adult eye. In the eye imaginal disc, however, a partial rescue of the *Ras* mutant phenotypes was observed with the Ras^{D38E} construct. In the presence of this transgene, the defect in growth was partially restored as measured by the increased ratio of the area occupied by the mutant and wild-type sister clone (Fig. 3). Furthermore, Ras^{D38E} cells survived behind the morphogenetic furrow and single Elav-positive cells were detected within the mutant clone (Fig. 4A,B), indicating that some mutant cells could differentiate neuronally. These single Elav-positive cells
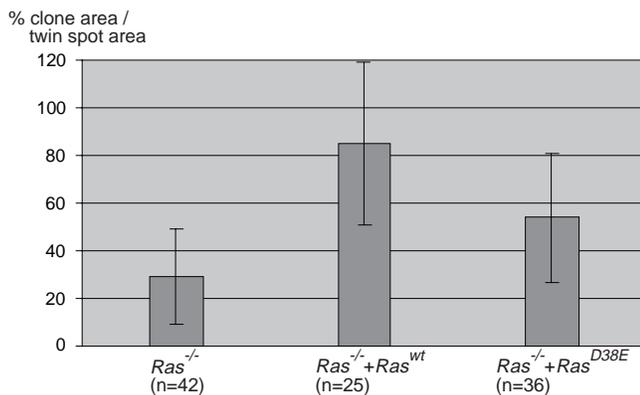

Fig. 3. Partial rescue of clone size by Ras^{D38E}. Clones of *Ras*^{x7b} were induced by heat shock at 48 hours AED and the effect on clonal growth was analyzed at 120 hours AED in the region of the prospective wing blade in the wing imaginal disc. The analysis was performed in the wing disc since divisions occur throughout the disc whereas in the eye disc the pattern of cell divisions changes along the anterior-posterior axis which renders a statistical analysis difficult. Bars represent mean values of the area of $Ras^{-/-}$ clones as percentage of their $Ras^{+/+}$ sister twin spots±s.d., *n*=number of $Ras^{-/-}$ and $Ras^{+/+}$ pairs analyzed. Expression of Ras^{wt} restored clone size to 85%. Ras^{D38E} partially increased the clone size. (Means of Ras^{wt} and Ras^{D38E} were significantly different from $Ras^{-/-}$, *t*-test: *P*<0.001.) Genotypes: left, *y w hsFLP; FRT82B Ras^{x7b}/FRT82B P(arm-lacZ)*; middle, *y w hsFLP;P(Ras^{wt},y$^+$)/+;FRT82B Ras^{x7b}/ FRT82B P(arm-lacZ)*; right, *y w hsFLP;P(Ras^{D38E},y$^+$)/+;FRT82B Ras^{x7b}/FRT82B P(arm-lacZ)*.

are R8 cells since they express the R8-specific marker Boss (Fig. 4C). Therefore the Ras^{D38E} mutant appears to provide sufficient Ras activity to prolong survival of postmitotic ommatidial cells and to support differentiation of R8 photoreceptors. Ras^{D38E} does not, however, provide enough activity to support recruitment of the other photoreceptor cells. No rescue of growth, cell survival and R8 differentiation was observed with the Ras^{Y40C} transgene (Fig. 4D,E).

The incomplete rescue observed with the Ras^{D38E} transgene may be due to insufficient activity of the Ras^{D38E} mutant protein towards its effector Raf. In mammalian cells, H-Ras^{G12V,D38E} leads to five times less activation of Raf than H-Ras^{G12V} (Rodriguez-Viciana et al., 1997). Alternatively, the partial rescue observed with Ras^{D38E} may be caused by its inability to activate a specific effector in addition to Raf. We therefore analyzed *Ras* mutant clones in the presence of multiple copies of Ras^{D38E} or a combination of Ras^{D38E} and Ras^{Y40C} mutant transgenes. Although we showed that Ras^{Y40C} does not activate PI3K, it may possess residual activity towards another, unidentified effector of Ras. However, neither increasing the copy number of the Ras^{D38E} transgene nor introducing a combination of the two different effector site mutants improved the rescue of $Ras^{-/-}$ photoreceptor cells in the adult (data not shown). This suggests that either the levels of Ras activity provided by up to three copies of the Ras^{D38E} transgene are insufficient to support differentiation of the R1-R7 photoreceptor cells or that Ras activity cannot be increased by simply augmenting the amount of mutant Ras protein in the cell. The specific activity of Ras^{D38E} may be too low because of a reduced binding affinity for Raf or an increased GTPase activity.

An increase in MAP kinase activity permits Ras^{D38E} to rescue cell survival and photoreceptor differentiation

To test whether the incomplete rescue of one or multiple copies of the Ras^{D38E} transgene is due to insufficient levels of MAP kinase activation or to the failure to activate additional effector pathways, we wanted to increase endogenous MAP kinase activity levels without rendering it independent of upstream activating signals. The *rolled^{Sem}* (*rl^{Sem}*) mutation is a dominant mutation in the gene encoding the *Drosophila* homolog of MAP kinase and causes an amino acid substitution (D334N) in the kinase domain (Brunner et al., 1994). This substitution renders MAP kinase partially resistant to dephosphorylation by MAPK phosphatases, which in turn results in a slightly elevated basal activity of MAP kinase and a prolonged activation (Cowley et al., 1994; Oellers and Hafen, 1996; Camps et al., 1998).

We assayed the ability of *rl^{Sem}* to assist Ras^{D38E} in promoting growth and differentiation in eyes in which Ras function was selectively removed. As loss of Ras function results in the death of postmitotic cells in the eye imaginal disc, such flies develop with a severely reduced head that almost completely lacks eyes (Fig. 5B). Introducing one copy of the *Ras* rescue construct completely restores eye size and structure (Fig. 5C). The Ras^{D38E} transgene causes a slight enlargement of the eye field owing to the presence of unpigmented cuticle (Fig. 5D, arrow); however, no additional ommatidial structures are formed. No significant increase in eye size was observed with the Ras^{Y40C} transgene. Similarly to Ras^{D38E}, the *rl^{Sem}*

Fig. 4. Ras^{D38E} supports survival and neuronal differentiation of R8 photoreceptor cells. Analysis of *Ras$^{-/-}$* clones in third instar eye imaginal discs of flies carrying the mutated *Ras* transgenes. *Ras* mutant clones were induced 24-48 hours AED by heat shock in a wild-type background (A,C,D), or in a *Minute* background, respectively (B,E). Discs were double labeled as described in Fig. 2 (β-gal, green, left panel; Elav, red, center panel; merged image, right panel), except for C, which was labeled with an antibody against Boss to detect R8 cells (green, left panel). Posterior is to the bottom. The *Ras^{D38E}* transgene rescues survival, differentiation, and regular spacing of single Elav-positive cells in *Ras$^{-/-}$* clones in a wild-type background (A) and in a *Minute* background (B). The single Elav-positive cells that are rescued by the *Ras^{D38E}* transgene in a *Ras$^{-/-}$* clone express the R8 marker Boss (C). In contrast to *Ras^{D38E}*, the *Ras^{Y40C}* transgene neither rescues R8 cell differentiation nor prolongs the survival of ommatidial cells posterior to the furrow in *Ras$^{-/-}$* clones in a wild-type background (D) nor in a *Minute* background (E), nor in two copies of *Ras^{Y40C}* (data not shown). The partial rescue of loss of Ras function by the *Ras^{D38E}* transgene is neither improved by two copies of *Ras^{D38E}* nor by a combination of *Ras^{D38E}* and *Ras^{Y40C}* transgenes (data not shown).
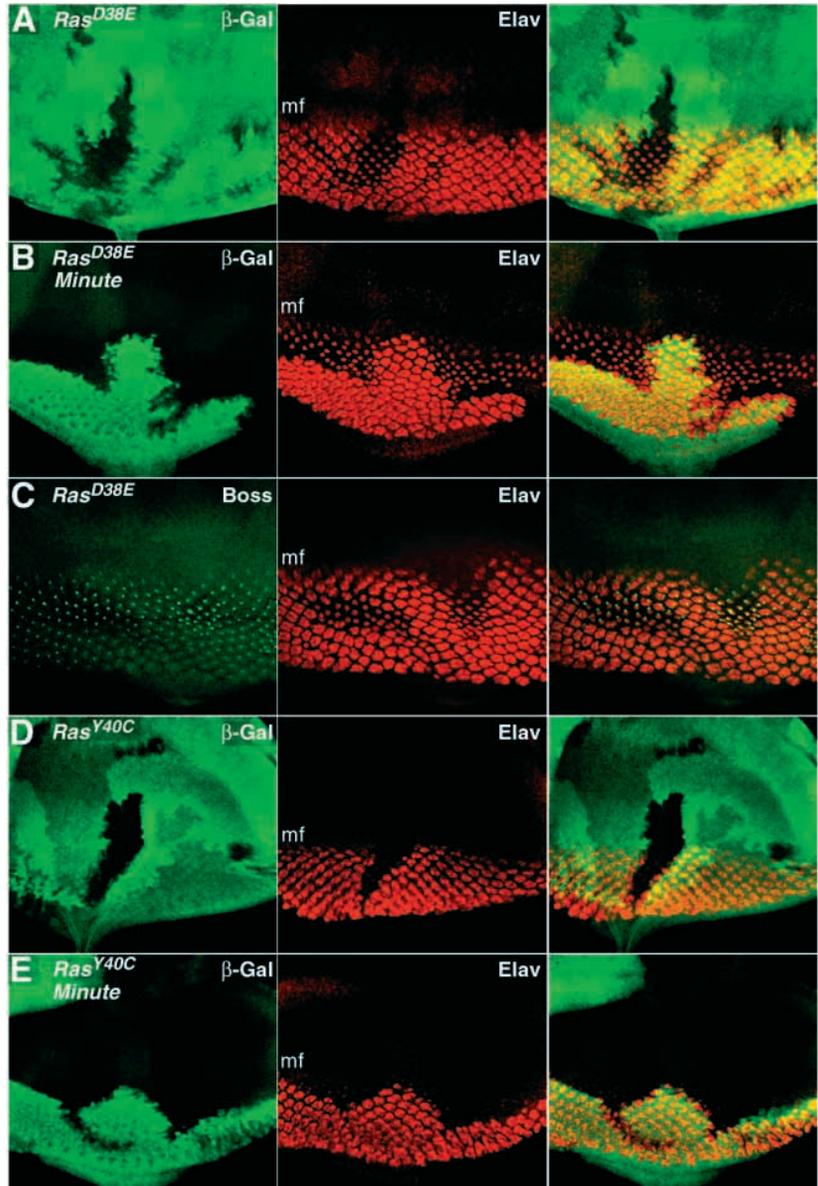
gain-of-function mutation alone was not sufficient for a significant increase in eye size (Fig. 5E), consistent with the biochemical and genetic evidence that this mutant protein is dependent on activation by Ras. The combination of *Ras^{D38E}* and *rl^{Sem}*, however, resulted in a substantial rescue of the eye and head size phenotype (Fig. 5F). Fully differentiated ommatidial structures are observed in these eyes. Histological sections through eyes of such flies reveal the presence of differentiated photoreceptor cells. A significant fraction of the ommatidia contained the full complement of photoreceptors (Fig. 5G). Therefore, the combination of *Ras^{D38E}* and *rl^{Sem}* is able to rescue R1-R7 differentiation and cell survival to adulthood. This indicates that the failure of Ras^{D38E} to specify R1-R7 cell fate and to promote survival of these cells in the adult eye can be compensated by increasing the basal activity of MAP kinase.

High levels of Ras and Raf activity are sufficient to induce precocious photoreceptor cell differentiation in the eye

Low levels of Ras activity are required for survival and differentiation of R8 cells and higher levels of Ras activity are needed for the differentiation of the R1-R7 cells. It has been shown that ectopic expression of activated EGF receptor or Ras promotes neuronal differentiation ahead of the morphogenetic furrow (Dominguez et al., 1998; Hazelett et al., 1998, and Fig. 6B). If our model according to which the levels of MAPK activity determine the Ras-mediated responses in the eye were correct, activated forms of Ras that can only activate Raf or activated Raf itself should also promote neuronal differentiation. We generated clones of cells expressing constitutively active wild-type and mutant forms of Ras and activated Raf in the eye imaginal disc using the FLP-out technique (Struhl and Basler, 1993). *Ras^{G12V}*-expressing clones located anterior to the morphogenetic furrow are rounded and express the neuronal maker Elav, indicating that these cells have undergone precocious neuronal development (Fig. 6B, arrow) and send out axonal projections. Thus, high levels of Ras activity are sufficient to induce neuronal differentiation in cells anterior to the morphogenetic furrow. Expression of Ras^{G12V,D38E} appeared equally efficient in inducing precocious neuronal differentiation as Ras^{G12V} in this assay (Fig. 6C, arrow). This is surprising as it is clearly less efficient in inducing ectopic R7 cells (Fig. 1C). As expected, Ras^{G12V,Y40C} was inactive (Fig. 6D). Similar to Ras^{G12V}, expression of an activated, membrane targeted version of Raf (Raf^{torY9}) was also sufficient to induce photoreceptor differentiation anterior to the furrow (Fig. 6E, arrow). As noted previously (Dominguez et al., 1998; Hazelett et al., 1998), the competence for neural differentiation in response to high Ras

or Raf activity is restricted to a zone anterior to the morphogenetic furrow. Clones in the antennal disc or in the periodial membrane do not undergo neuronal differentiation. We conclude that in cells within the competence zone in the eye imaginal disc, high levels of Ras or Raf activity are necessary and sufficient to induce neuronal differentiation. In the same cells, low levels of Ras activity promote growth. The regulation of precise levels of Ras activity during eye development is therefore important for the correct cellular response.

DISCUSSION

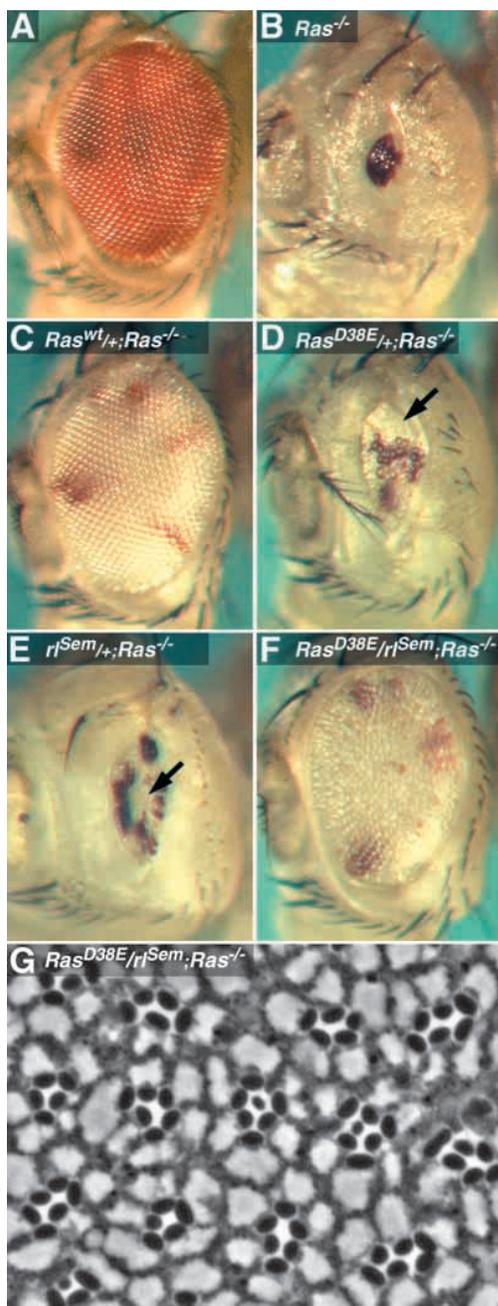We have presented evidence that Ras signaling controls

Fig. 5. Increasing MAP kinase activity permits Ras^{D38E} to rescue cell survival and photoreceptor differentiation. To test the rescue of *Ras^{D38E}* in a background with increased MAPK activity we selectively removed Ras function in eye imaginal disc cells using the eyFLP, cell lethal technique (Newsome et al., 2000). The mutant tissue is marked by the absence of pigment. Genotypes: (A) *eyFLP/+;FRT82B w$^+$ cl3R3/TM3*; (B) *eyFLP/+; FRT82B Ras^{x7b}/ FRT82B w$^+$ cl3R3*; (C) *eyFLP/+; P(Ras^{wt},y$^+$)/+; FRT82B Ras^{x7b}/ FRT82B w$^+$ cl3R3*; (D) *eyFLP/+; P(Ras^{D38E},y$^+$)/+; FRT82B Ras^{x7b}/ FRT82B w$^+$ cl3R3*; (E) *eyFLP/+; rl^{Sem}/+; FRT82B Ras^{x7b}/ FRT82B w$^+$ cl3R3*; (F,G) *eyFLP/+; P(Ras^{D38E},y$^+$)/rl^{Sem}; FRT82B Ras^{x7b}/ FRT82B w$^+$ cl3R3*. *eyFLP/+;FRT82B w$^+$ cl3R3/TM3* flies show wild-type eyes (A). Selective removal of Ras function in the eye by *eyFLP/+;FRT82B Ras^{x7b}/FRT82B w$^+$ cl3R3*, generates flies with a drastically reduced head capsule and reduced eyes (B). One copy of the *Ras* rescue construct restores the head and the eye size (C). The *Ras^{D38E}* transgene (D) or the *rl^{Sem}* mutation (E) alone do not rescue the eye structure, but eye size is slightly increased due to undifferentiated cuticle in the eye (arrows). The *Ras^{D38E}* transgene in a *rl^{Sem}* background rescues *Ras$^{-/-}$* photoreceptors (F). Tangential section through an eye shown in (F) reveals the presence of differentiated R1-R7 photoreceptor cells (G).

different cellular responses by at least two thresholds of MAPK activity in the eye imaginal disc of *Drosophila*. Low levels of MAPK activity permit growth, survival of postmitotic cells and R8 differentiation, while high activity induces R1-R7 differentiation. These data significantly extend results from previous studies on the role of EGFR function during eye development. It provides direct evidence that, under physiological conditions not involving overexpression, altering the levels of a single effector pathway, the Raf/MAPK pathway, is sufficient to elicit distinct cellular responses.

Ras and growth control

Ras was first identified as an oncogene in vertebrates (reviewed by Bourne et al., 1990). Studies in tissue culture cells have suggested that the primary role of Ras is in the control of cell proliferation, as the mitogenic response to a variety of growth factors can be blocked by inhibiting *Ras* function (Mulcahy et al., 1985; Smith et al., 1986). However, we show that in the eye imaginal disc, cells proliferate in the absence of Ras, albeit with a reduced growth rate, implying that Ras is not essential for proliferation in this system. The reason for the small size of *Ras* mutant clones compared to their twin spots is not only the intrinsic growth deficit of these cells but is caused by their failure to compete successfully with the faster growing wild-type cells. These results are in agreement with a recent study of *Ras* function in the wing disc (Prober and Edgar, 2000).

How does Ras control growth? One possibility is that Ras directly binds and activates PI3K. Clones mutant for components in the insulin receptor/PI3K pathway also have a growth disadvantage compared to wild-type cells (Böhni et al., 1999; Montagne et al., 1999; Weinkove et al., 1999). Although in vertebrates, H-Ras^{G12V,Y40C} activates PI3K, we found no evidence that the corresponding mutant activates PI3K in *Drosophila*. Partial loss-of-function mutations in genes coding for Raf and MAPK, respectively, showed similar growth defects as *Ras* mutants (Diaz-Benjumea and Hafen, 1994). Furthermore, Ras^{D38E} showed a significant rescue of the growth disadvantage of *Ras$^{-/-}$* clones. Thus, we propose that

while cell growth depends on the activities of the MAP kinase as well as the PI3K pathway, the activation of the MAP kinase pathway is only Ras-dependent. It has recently been proven that cooperation between the Ras/MAP kinase and the PI3K/PKB pathway is required in order to induce growth in cultured cells. In fibroblasts, activation of Raf and PI3K is required for cyclin D1 expression and entry into S-phase (Gille and Downward, 1999). Induction of DNA synthesis by activation of the platelet-derived growth factor (PDGF) receptor requires an early activation of MAPK and a late phase PI3K activity (Jones et al., 1999).

The role of Ras in survival and differentiation

Ras mutant cells located behind the morphogenetic furrow die by programmed cell death (PCD). Ras controls the PCD inducer Hid, by repressing its expression and by modifying its activity through phosphorylation by MAPK (Bergmann et al., 1998; Kurada and White, 1998). In mammalian cells, PI3K promotes survival via PKB-mediated phosphorylation of the pro-apoptotic protein Bad. Thus, survival could at least in part be mediated by the activation of PI3K. Indeed, a partial suppression of Hid-induced apoptosis in the eye by the expression of $Ras^{G12V,Y40C}$ was taken as evidence that PI3K supports survival in the developing eye (Bergmann et al., 1998). This is unlikely in the light of the data presented here, as we have shown that $Ras^{G12V,Y40C}$ is unable to activate PI3K. Furthermore, the Ras^{D38E} transgene rescues $Ras^{-/-}$ cells posterior to the morphogenetic furrow from PCD. Thus it appears that the function of Ras in survival is mediated exclusively through the activation of MAPK. In the adult, however, *Ras* mutant cells were never observed. This may be due to an exclusive role of Ras in promoting cell survival of ommatidial cells at later stages or due to an additional role in cell fate specification. Several lines of evidence argue against an exclusively anti-apoptotic role of Ras during the later stages of eye development. First, reduced Ras activity in the R7 precursor cell in the absence of the Sev receptor tyrosine kinase results in a change in cell fate rather than death of this progenitor cell (Tomlinson and Ready, 1986). Second, constitutive activation of Ras in cone cell precursors is sufficient to induce R7 differentiation in these cells (Fortini et al., 1992). Third, ectopic expression of an activated EGF receptor or Ras^{G12V} results in precocious induction of photoreceptor cell differentiation anterior to the furrow (Hazelett et al., 1998; Domínguez et al., 1998 and this study). Thus in the case of R1-R7 differentiation, high levels of Ras activity are required for a choice in cell fate rather than mere survival of the cells. The differentiation of the R8 cells, which we have shown to depend on Ras activity, however, may be different. R8 cell differentiation is rescued by Ras^{D38E}, concomitant with the survival of the mutant clones. Therefore it is possible that Ras-mediated survival is sufficient for R8 cell differentiation. Interestingly, loss of EGF receptor function still allows the formation of R8 cells (Domínguez et al., 1998), suggesting that the low levels of Ras activity
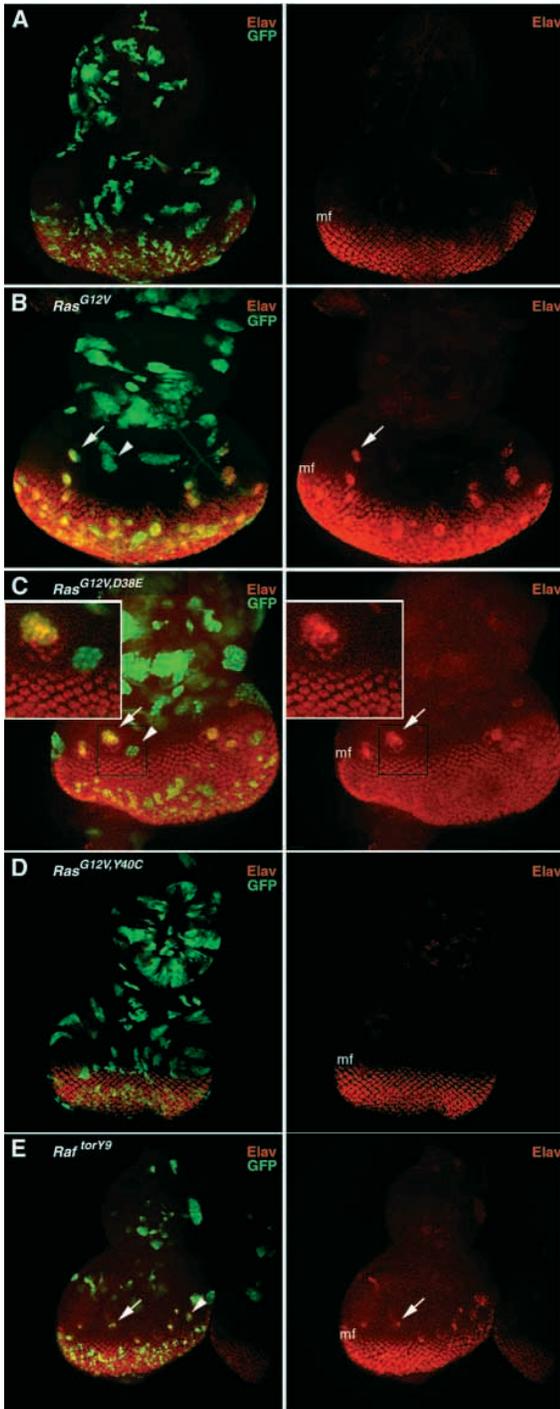
Fig. 6. High levels of Ras activity induces neuronal differentiation. Analysis of clones expressing activated Ras in third instar eye imaginal discs. Clones of cells expressing *UAS-Ras^{G12V}* under Act-Gal4 control were induced 48-72 hours AED by heat shock. The clone is visualized by co-expression of GFPnls (green) and neuronal differentiation is visualized by labeling with an antibody to the nuclear neuronal marker Elav (red, right panel). The merged image is shown in the left panel. Posterior is towards the bottom. Wild-type clones are shown as a control in (A). Clones expressing *Ras^{G12V}* round up and express the neuronal marker Elav anterior to the morphogenetic furrow (B, arrow). Expression of *Ras^{G12V,D38E}* is sufficient to induce the neuronal marker Elav in clones in front of the furrow (C, arrow, the inset shows a magnification of a *Ras^{G12V,D38E}* clone in front of the furrow), whereas expression of *Ras^{G12V,Y40C}* does not induce Elav in front of the furrow (D). *Raf^{torY9}* expression in front of the furrow is sufficient to induce Elav expression (E, arrow). Clones of activated Ras or Raf in the peripodial membrane do not express Elav (arrowheads).
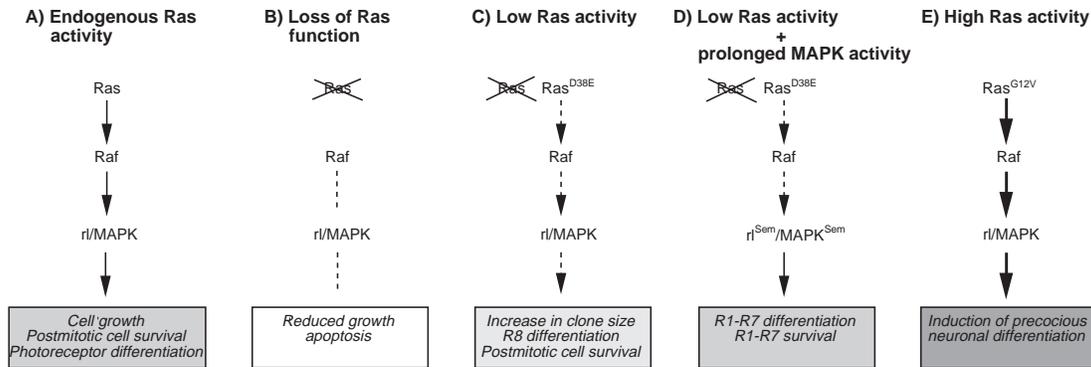
Fig. 7. Different thresholds of a single Ras effector pathway specify distinct cellular responses within the same cells. Our current model for Ras function in *Drosophila* eye development. (A) Ras is required for growth, cell survival and photoreceptor differentiation. These responses are mediated via Raf/MAP kinase signaling. (B) In the absence of Ras, mutant cells grow at a reduced rate, do not differentiate and die upon exit from the cell cycle. (C) Low levels or transient activation of MAPK achieved by Ras^{D38E} support cell survival and R8 differentiation. (D) Elevated levels or prolonged activation of MAPK achieved by combining Ras^{D38E} with the inactivation-resistant form of MAPK (Rl^{Sem}) rescue R1-R7 photoreceptor differentiation and survival to the adult eye. (E) High levels or persistent activation by Ras^{G12V} or Ras$^{G12V, D38E}$ in dividing cells lead to precocious photoreceptor differentiation.

required for R8 differentiation are achieved by another receptor system.

Different thresholds of Ras/MAPK activity induce distinct cellular responses in the developing eye

There are three different models for how specificity of Ras signaling is achieved: Specificity may be controlled by (1) the cellular context, (2) the activation of distinct signaling pathways by Ras or (3) by different levels of Ras activity. The experiments presented here support the importance of the cellular context and the different levels of Ras activity but fail to provide evidence for the activation of different signaling pathways by Ras. All aspects of Ras signaling could be rescued by the activation of the Ras/MAPK pathway and we found no evidence, using the Ras effector site mutants, that constitutively active Ras activates the PI3K pathway directly.

The cellular context in which Ras activates MAP kinase is clearly important. Expression of Ras^{G12V} in blastoderm cells triggers differentiation of head and tail structures (Melnick et al., 1993; Greenwood and Struhl, 1997), it triggers vein differentiation in wing disc cells (Prober and Edgar, 2000) and neuronal differentiation in eye disc cells. We have shown here that different levels of Ras/MAPK activity appear to control distinct cellular responses within the same tissue (Fig. 7). Low levels of Ras activity, provided by the *Ras^{D38E}* mutant, rescue R8 differentiation and survival but not R1-R7 differentiation. High levels of Ras/MAPK activity provided by wild-type Ras or by a combination of *Ras^{D38E}* and *rl^{Sem}* are required for the differentiation of R1-R7 photoreceptor cells.

There are two possibilities with regard to the nature of the activity thresholds that elicit the different cellular responses. The threshold may be quantitative. Cells could react to different activity levels within the cells. Alternatively, the threshold may be temporal and cells react to the difference in the duration of the signal. Staining of imaginal discs with an antibody that selectively recognizes activated MAPK (dpERK, Gabay et al., 1997) was not sensitive enough to detect activated MAPK during normal photoreceptor cell recruitment or during ectopic neuronal differentiation in Ras^{G12V}-expressing clones anterior to the morphogenetic furrow (data not shown).

Therefore, we cannot distinguish between these two models. In the present case, however, we favor the temporal model because we do not detect the highest levels of dpERK staining behind the morphogenetic furrow during photoreceptor cell recruitment. In response to MAP kinase activation in the developing eye, a number of negative regulators of the pathway are induced. The EGF-related peptide Argos competes with the TGFα-like ligand Spitz for EGF receptor binding (Freeman, 1994; Golembo et al., 1996), and Sprouty, a cytoplasmic protein, associates with the EGF receptor to turn off the signaling pathway (Casci et al., 1999). Indeed, neuronal differentiation and ommatidial development in the *Ras^{D38E}* mutant is rescued by the prolonged activity of MAPK caused by the *rl^{Sem}* mutation. It is possible that the reduced activity of Ras^{D38E} towards Raf is caused by a more rapid inactivation, owing to increased GTPase activity. The observation that Ras^{G12V,D38E} is sufficient to induce neuronal differentiation ahead of the furrow, in conjunction with the G12V substitution, which inactivates the Ras GTPase activity is consistent with the idea that D38E may stimulate GTP hydrolysis. Thus, neuronal differentiation in *Drosophila* may depend on the prolonged activation of Ras/MAP kinase, whereas transient activation is sufficient for survival upon exit from the cell cycle and differentiation of R8 photoreceptor cells. Therefore it appears that neuronal differentiation in response to Ras activation in the developing eye of *Drosophila* is similar to neuronal differentiation in PC12 cells, which also requires prolonged activation of MAPK (Marshall, 1995). The modulation of levels and/or the duration of Ras/MAPK activity levels appear to be important determinants of cellular responses in multicellular organisms.

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