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The *Drosophila rolled* locus encodes a MAP kinase required in the sevenless signal transduction pathway

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Mitogen-activated protein (MAP) kinases have been proposed to play a critical role in receptor tyrosine kinase (RTK)-mediated signal transduction pathways. Although genetic and biochemical studies of RTK pathways in *Caenorhabditis elegans*, *Drosophila melanogaster* and mammals have revealed remarkable similarities, a genetic requirement for MAP kinases in RTK signaling has not been established. During retinal development in *Drosophila*, the sevenless (Sev) RTK is required for development of the R7 photoreceptor cell. Components of the signal transduction pathway activated by Sev in the R7 precursor include proteins encoded by the *gap1*, *drk*, *Sos*, *ras1* and *raf* loci. In this report we present evidence that a *Drosophila* MAP kinase, ERK-A, is encoded by the *rolled* locus and is required downstream of *raf* in the Sev signal transduction pathway.

Key words: Drosophila/MAP kinase/receptor tyrosine kinase/signal transduction pathways

Introduction

The mitogen-activated protein (MAP) kinases comprise a family of serine/threonine protein kinases that are activated in response to a wide array of extracellular signals (e.g. epidermal growth factor, nerve growth factor and insulin) transduced by receptor tyrosine kinases (RTKs) (Pelech and Sanghera, 1992; Blenis, 1993). Biochemical and cell culture studies have revealed that RTK-mediated activation of MAP kinase requires a core signal transduction pathway that includes Grb2/sem5/Drk, Sos, Ras, Raf and Mek. The order of action of these proteins has been determined through the use of dominant negative and constitutively active forms of these proteins and by the demonstration of direct protein–protein interactions. For instance, the activation of Raf in cell lines expressing a constitutively active form of Ras (e.g. Ras^{Val12}) indicated that Ras acts upstream of Raf in the RTK-activated signaling pathway (Morrison *et al.*, 1988; Kolch *et al.*, 1991). Recent experiments indicate that a direct interaction between Ras and Raf is necessary for Raf activation (Moodie *et al.*, 1993; Vojtek *et al.*, 1993; Xhang *et al.*, 1993). The ability of a dominant negative form of Ras (Ras^{S17N}), expressed in the PC12 pheochromocytoma cell line or in fibroblasts, to block the activation of both Raf and MAP kinase after growth factor treatment provides additional evidence that Ras acts upstream of both Raf and MAP kinase (Thomas *et al.*, 1992; Wood *et al.*, 1992). Finally, growth factor-independent activation of MAP kinase by constitutively activated forms of Ras or Raf demonstrates that MAP kinase acts downstream of both Ras and Raf in the RTK-activated signaling pathway (Dent *et al.*, 1992; Howe *et al.*, 1992).

The activation of MAP kinase requires phosphorylation of threonine and tyrosine residues by a unique dual-specificity kinase termed Mek (MAP or ERK kinase) (Ahn *et al.*, 1992; Crews and Erikson, 1993). The ability of Raf to phosphorylate and activate MEK indicates that MEK provides the link between Raf and MAP kinase activation. The translocation of activated MAP kinase to the nucleus supports a role for MAP kinase in the regulation of nuclear proteins such as transcription factors (Chen *et al.*, 1992; Gonzalez *et al.*, 1993; Lenormand *et al.*, 1993). The targets of MAP kinase activity include the pp90 ribosomal S6 kinase ($pp90^{RSK}$) (Blenis, 1993), as well as the transcription factors c-Myc (Seth *et al.*, 1992), c-Jun (Pulverer *et al.*, 1991, 1993) and the *ets*-domain-containing protein Elk-1 (Gille *et al.*, 1992; Hill *et al.*, 1993; Marais *et al.*, 1993). In each of these instances, phosphorylation by MAP kinase has been shown to result in the activation of the target protein.

Although extensive biochemical analysis suggests that MAP kinase provides a critical link between signal transduction in the cytoplasm and changes in gene expression in the nucleus, a genetic requirement for MAP kinase function in signaling pathways activated by RTKs has not, as yet, been demonstrated. As an initial step in the genetic analysis of MAP kinase function in RTK signaling, we previously identified a homolog of MAP kinase, ERK-A, expressed throughout development in *Drosophila melanogaster* (Biggs and Zipursky, 1992). In this paper, we assess a genetic requirement for MAP kinase activity in the sevenless (Sev) signal transduction pathway during development of the compound eye.

The compound eye of *Drosophila* is composed of some 800 simple eyes called ommatidia (Figure 1a and d) (recently reviewed in Cagan and Zipursky, 1992). Each ommatidium contains eight photoreceptor neurons, designated R1–R8, and 12 non-neuronal cells. The development of the R7 cell requires an inductive interaction between the Sev RTK, located on the surface of the R7 precursor cell, and the Bride of sevenless (Boss) protein expressed on the surface of the neighboring R8 cell (Hafen *et al.*, 1987; Reinke and Zipursky, 1988; Krämer *et al.*, 1991; Hart *et al.*, 1993). *Drosophila* Ras1, putative regulators of Ras1 (Gap1, Sos and Drk), and the serine/threonine kinase Raf have all been shown to be components of the Sev signal transduction pathway (Rogge *et al.*, 1991, 1992; Simon *et al.*, 1991,

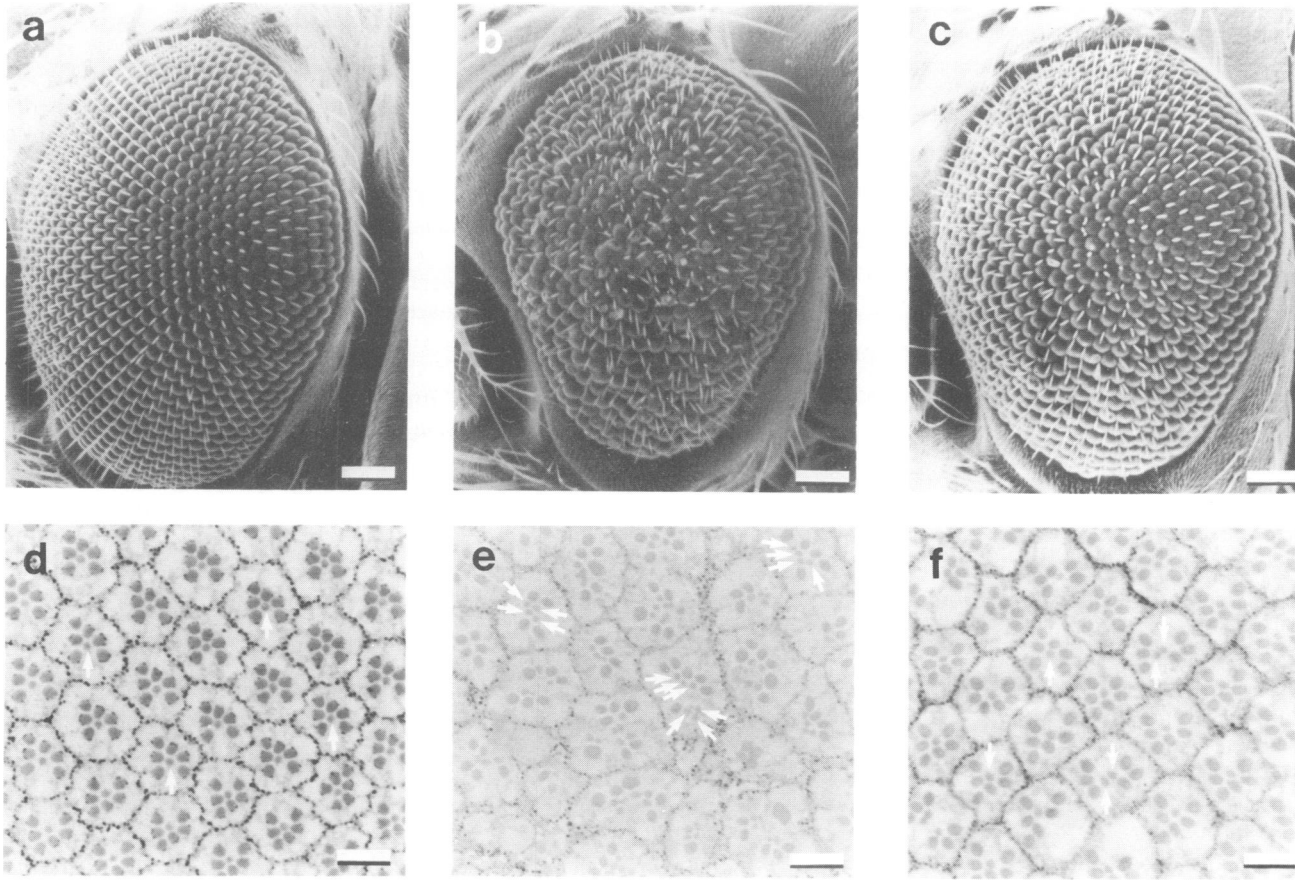

Fig. 1. The *rl* gene is required in the Sev signal transduction pathway. (**a** and **d**) Normal adult eyes. The small rhabdomere (light-sensitive organelle) profile of the R7 cell is indicated by the arrows. (**b** and **e**) Flies, expressing a dominantly active form of the serine-threonine kinase *raf* under the control of the *sev* enhancer (sE-raf^{torY9}/+) in a set of cells referred to as the R7 equivalence group, have rough eyes due to the development of additional R7 cells (arrows). (**c** and **f**) The rl^{4H6} mutation was isolated as a dominant suppressor of raf^{torY9}. As shown, the rough eye phenotype caused by raf^{torY9} is suppressed in flies heterozygous for rl^{4H6} (rl^{4H6}/+, sE-raf^{torY9}). Note the reduction in the number of additional R7 cells (arrows). (a–c) Scanning electron micrographs of adult eyes. Scale bars = 43 μm. (d–f) Plastic sections of adult eyes visualized by light microscopy. Scale bars = 9 μm.

1993; Bonfini *et al.*, 1992; Buckles *et al.*, 1992; Dickson *et al.*, 1992b; Gaul *et al.*, 1992; Olivier *et al.*, 1993). A *Drosophila* homolog of Mek, Dsor1, has recently been shown to act downstream of *raf* in the Torso RTK signaling pathway during embryogenesis (Tsuda *et al.*, 1993). A requirement for Dsor1 in the Sev signal transduction pathway has not been assessed.

During normal development, the R7 inductive pathway is activated in a single cell within each ommatidium, the R7 precursor cell. However, expression of constitutively active forms of Sev, Ras1 or Raf in a group of cells within each developing ommatidium, referred to as the R7 equivalence group (i.e. the R7 and cone cell precursors, as well as a cell of unknown fate, the so-called 'mystery cell'), results in the development of additional R7 cells (Basler *et al.*, 1991; Dickson *et al.*, 1992a; Fortini *et al.*, 1992). This leads to a readily visible 'rough eye' phenotype (see below). In cases where the level of a downstream component is limiting, a reduction in gene dosage should attenuate the signal and reduce the severity of the 'rough eye' phenotype. Biochemical studies in vertebrates as well as epistasis studies in *Drosophila* have shown that Raf lies downstream of both Sev and Ras1 (Dickson *et al.*, 1992b; Vojtek *et al.*, 1993; Xhang *et al.*, 1993), making Raf a useful stepping stone for the identification of proteins acting later in the pathway. In this paper we report that mutations in the *Drosophila* MAP kinase, ERK-A, suppress a constitutively activated form of *raf* expressed in the R7 equivalence group (sE-raf^{torY9}) thereby establishing a role for *Drosophila* MAP kinase in the Sev signal transduction pathway.

Table I. The *rl* dependence of the R7 inductive pathway was assessed in the sensitized genetic background ($w,sev^{E4};Sos^{JC2}$/+)[a]

Genotype	% Ommatidia containing R7
$w,sev^{E4};Sos^{JC2}+/Sos^{JC2}+;+/+$	41 (n = 561)
$w,sev^{E4};++/Sos^{JC2}+;+/+$	27 (n = 958)
$w,sev^{E4};+rl^{1}/Sos^{JC2}+;+/+$	1.2 (n = 1517)

[a]Rogge *et al.* (1991).
The rl^{1} allele was isolated as a spontaneous mutation (Morgan *et al.*, 1925).

Results

Requirement for rolled (rl) during R7 development

To isolate genes acting downstream of Raf, a mutagenesis was carried out to identify dominant enhancers and suppressors of the rough eye phenotype caused by

Table II. *ERK-A ^{WT}* rescues lethality of *rl* mutants

Genotype	Heat shock	Number of progeny
rl^{4H6}/Df(2R)rl^{10a};2xsE-ERK-A^{WT}	–	<0.7% (0/139)
rl^{4H6}/Df(2R)rl^{10a};2xsE-ERK-A^{K164}	–	<0.7% (0/138)
rl^{4H6}/Df(2R)rl^{10a};2xsE-ERK-A^{WT}	+	30.4% (51/168)
rl^{4H6}/Df(2R)rl^{10a};2xsE-ERK-A^{K164}	+	<0.8% (0/120)

Individuals carrying a heteroallelic combination of two different null alleles, *rl^{4H6}* and *Df(2R)rl^{10a}*, die as larvae. Repetitive heat shock-induced expression of *ERK-A^{WT}*, but not *ERK-A^{K164}*, throughout development rescued this phenotype. In these crosses, 25% of the adults should carry *rl^{4H6}/Df(2R)rl^{10a}*; heat shock-induced expression of ERK-A results in near quantitative rescue of lethality.

expression, within the R7 equivalence group, of *raf^{torY9}*, a constitutively active form of Raf (Dickson *et al.*, 1992b; B.Dickson, A.van der Straten and E.Hafen, unpublished data) (Figure 1b and e). Several mutations found to suppress *raf^{torY9}* proved to be alleles of the previously identified *rolled* locus (Figure 1c and f) (Morgan *et al.*, 1925; Hilliker, 1976). These *rl* alleles also suppressed the rough eye phenotype generated by expression, within the R7 equivalence group, of *ras1^{val12}*, a constitutively active form of Ras1 (Brunner *et al.*, 1994). Additional alleles of *rl* were isolated independently as dominant suppressors of *ras1^{val12}* (Fortini *et al.*, 1992; H.Chang, F.Karim, D.Wassarman, M.Therrien and G.Rubin, personal communication).

A function for *rl* in R7 development was also demonstrated by a dosage-dependent enhancement of a weak *sev* phenotype. Approximately 27% of the ommatidia from male flies hemizygous for the weak loss-of-function allele *sev^{E4}* and heterozygous for the gain-of-function *Sos^{JC2}* allele (*sev^{E4};Sos^{JC2}/+*) contained an R7 cell (Rogge *et al.*, 1991). A reduction in *rl* activity in the *sev^{E4};Sos^{JC2}* background, caused by removing one copy of the *rl* gene, resulted in a 23-fold decrease (from 27 to 1.2%) in the percentage of ommatidia containing R7 cells (Table I). A reversal in the suppression of *sev^{E4}* by *Sos^{JC2}* has also been observed upon reduction in gene dosage of other members of the Sev signal transduction pathway (Rogge *et al.*, 1991; Bonfini *et al.*, 1992). These studies indicate that *rl* is required for R7 induction and that it acts downstream of, or in parallel with, Raf.

The function of *rl* in the compound eye is not restricted to R7 development. Flies homozygous for the weak *rl* allele, *rl^{1}*, have rough eyes. Mutant ommatidia from these eyes invariably contained fewer R cells, with the development of the R7 cell appearing to be most sensitive to reduction in *rl* activity (data not shown). Flies homozygous for *rl* null mutations die as larvae and show a significant reduction in the size of the imaginal discs, reflecting a defect in cellular proliferation and/or survival (Hilliker, 1976; Dimitri, 1991). These data indicate that *rl* plays a widespread role in development.

Expression of the MAP kinase ERK-A complements rl mutations

The *rl* locus maps to the heterochromatic region of chromosome 2R (Hilliker, 1976). A previously cloned *Drosophila* MAP kinase (ERK-A), highly homologous to both yeast and vertebrate MAP kinases, was also mapped to this region of the second chromosome by *in situ* hybridization (Biggs and Zipursky, 1992, 1993). Since biochemical studies in vertebrates placed MAP kinases downstream of p21$^{c\text{-}ras}$ (Thomas *et al.*, 1992; Wood *et al.*, 1992), as well as *c*-raf (Howe *et al.*, 1992; Kyriakis *et al.*, 1992; Williams *et al.*, 1993), we set out to determine if the *rl* locus encodes ERK-A. Due to the large size (~100 kb) of the *ERK-A* gene (W.H.Biggs, K.H.Zavitz and S.L.Zipursky, unpublished observation), genomic rescue of *rl* mutants was not feasible. As an alternative approach, transgenic flies were generated which expressed either active (*ERK-A^{WT}*) or inactive (*ERK-A^{K164}*) forms of the ERK-A protein under the control of both the *sev* enhancer, driving expression within the R7 equivalence group, and the heat shock-inducible *hsp70* promoter. Periodic heat shock-induced expression of *ERK-A^{WT}* throughout development rescued the larval lethality of *rl* null alleles (Table II). In addition, the expression of *ERK-A^{WT}* reversed the suppression of the rough eye phenotype of *raf^{torY9}* (Figure 2a–f) and the enhancement of *sev^{E4};Sos^{JC2}* phenotype (Table III) caused by a reduction in *rl* activity. The mutant *ERK-A^{K164}* failed to complement *rl* mutations.

Molecular lesions in ERK-A found in rl alleles

Using protein immunoblots, the levels of ERK-A protein in two X-ray and five ethylmethane sulfonate-induced alleles were assessed (Figure 3a). This was done in heterozygous adults since no appropriate controls were available for homozygous larvae. All seven alleles showed a reduction in ERK-A protein levels (35 to 57% of wild-type), consistent with a 2-fold decrease in gene dosage (see legend to Figure 3 for details of quantitation). In addition to exhibiting reduced levels of full-length ERK-A, a weak immunoreactive band with a decrease in apparent molecular weight of 3–4 kDa was observed in homogenates of *rl^{4H6}* heterozygotes. This lower molecular weight band is shown in larvae homozygous for the *rl^{4H6}* allele (Figure 3b). Sequencing of an ERK-A cDNA generated by RT-PCR from the *rl^{4H6}* mutant revealed that this difference in molecular weight is due to a 135 bp (45 amino acid) in-frame deletion corresponding to kinase subdomain VI, a region highly conserved between different MAP kinases (Figure 3c and d) (Hanks *et al.*, 1988). The deleted region corresponds precisely to exon 4 of the *ERK-A* gene (W.H.Biggs, K.H.Zavitz and S.L.Zipursky, unpublished observations); the DNA lesion leading to this difference in splicing was not determined. An additional allele, *rl^{64}*, (Hilliker, 1976) contains a nonsense mutation at codon 353 that is predicted to result in a C-terminal truncation of 24 amino acids (Figure 3d). The remaining five alleles were not sequenced.

Discussion

The rolled locus encodes a Drosophila MAP kinase required during R7 development

Three lines of evidence establish that *rl* encodes a *Drosophila* MAP kinase required in the Sev signal transduction pathway (Figure 4). First, *rl* alleles were identified as dosage-sensitive suppressors of a constitutively activated form of *raf* expressed in the R7 equivalence group (sE-*raf^{torY9}*). Furthermore, a reduction in *rl* activity also reversed the suppression of *sev^{E4}* by *Sos^{JC2}*. These results indicate that *rl* acts downstream of *raf* in the Sev signaling pathway. Second, molecular analysis revealed that two alleles of the *rl* locus,

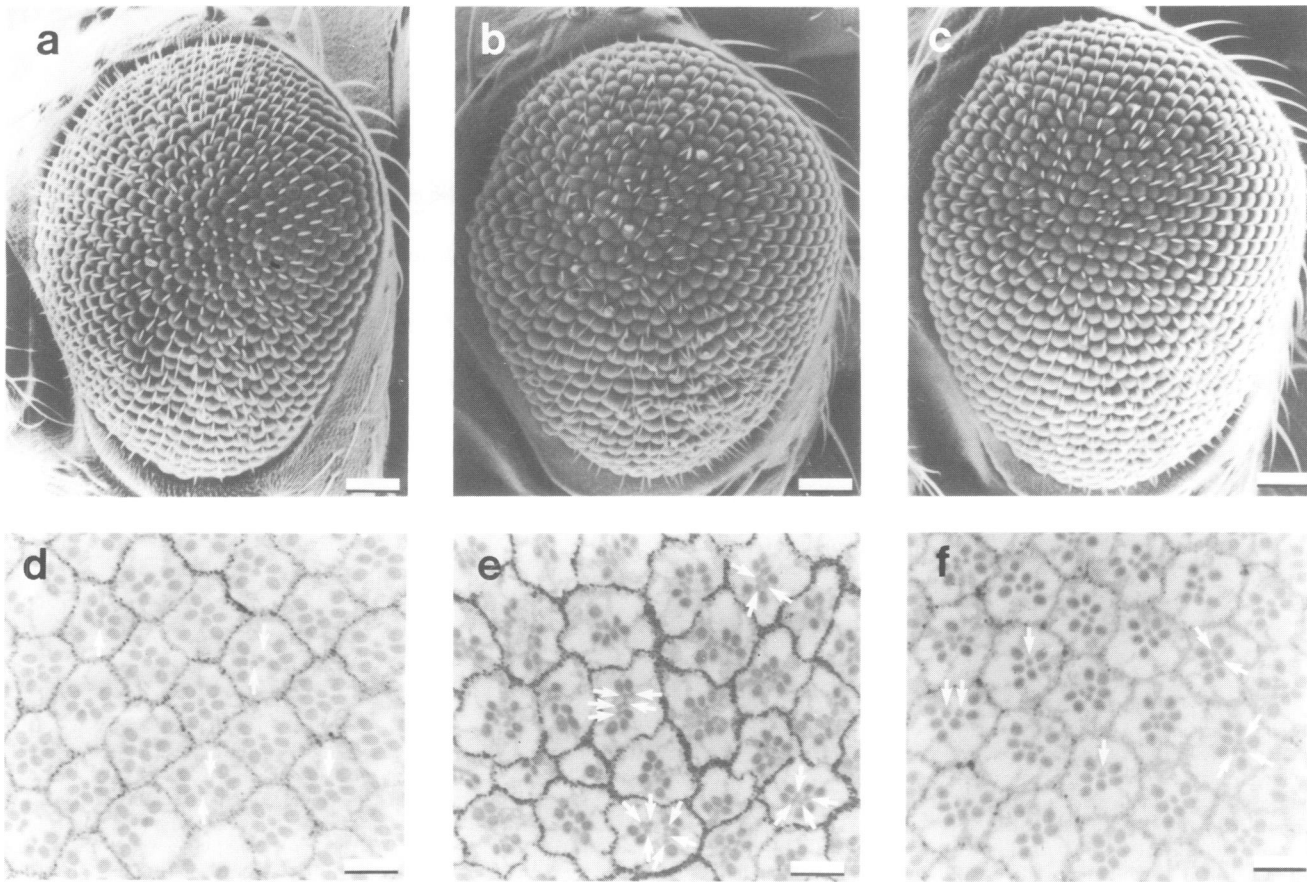

Fig. 2. The *ERK-A^{WT}* transgene complements *rl* mutations. (**a** and **d**) The rough eye phenotype caused by *raf^{torY9}* is suppressed in flies heterozygous for *rl^{4H6}* (*rl^{4H6}*/+, sE-*raf^{torY9}*). These panels are the same as shown in Figure 1c and f. (**b** and **e**) The *rl* suppression of sE-*raf^{torY9}* is partially reversed by the presence of an *ERK-A^{WT}* transgene under the control of two copies of the sev enhancer (2xsE-*ERK-A^{WT}*). The genotype of the eyes shown is *rl^{4H6}*/+, sE-*raf^{torY9}*;2xsE-*ERK-A^{WT}*. (**c** and **f**) Expression of the catalytically inactive *ERK-A^{K164}* under a tandem repeat of the *sev* enhancer (2xsE-*ERK-A^{K164}*) does not reverse the suppression of sE-*raf^{torY9}* by *rl*. The genotype of the eyes shown is *rl^{4H6}*/+, sE-*raf^{torY9}*;2xsE-*ERK-A^{K164}*. (a–c) Scanning electron micrographs of adult eyes. Scale bars = 43 μm. (d–f) Plastic section of adult eyes visualized by light microscopy. Arrows indicate the position of R7 cells. Scale bars = 9 μm.

Table III. The *rl* suppression of the *sev^{E4}*;*Sos^{JC2}*/+ phenotype (see Table I) is reverted by the 2xsE-*ERK-A^{WT}* transgene

Genotype	% Ommatidia containing R7
w,*sev^{E4}*;*Sos^{JC2}*+/*Sos^{JC2}*+;+/+	41 (*n* = 561)
w,*sev^{E4}*;++/*Sos^{JC2}*+;+/+	27 (*n* = 958)
w,*sev^{E4}*;+*rl^{1}*/*Sos^{JC2}*+;+/+	1.2 (*n* = 1517)
w,*sev^{E4}*;+*rl^{1}*/*Sos^{JC2}*+;2xsE-*ERK-A^{WT}*/+	8 (*n* = 5173)
w,*sev^{E4}*;+*rl^{1}*/*Sos^{JC2}*+;2xsE-*ERK-A^{WT}*/2xsE-*ERK-A^{WT}*	22 (*n* = 1151)
w,*sev^{E4}*;+*rl^{1}*/*Sos^{JC2}*+;2xsE-*ERK-A^{K164}*/+	1.6 (*n* = 1671)
w,*sev^{E4}*;+*rl^{1}*/*Sos^{JC2}*+;2xsE-*ERK-A^{K164}*/2xsE-*ERK-A^{K164}*	0.0 (*n* = 1229)

One copy of 2xsE-*ERK-A^{K164}* did not revert the *rl* suppression of the *sev^{E4}*;*Sos^{JC2}* phenotype, while two copies further enhanced the *sev* phenotype.

rl^{4H6} and *rl^{64}*, contain mutations leading to internally deleted and C-terminally truncated forms of ERK-A, respectively. Finally, expression of the ERK-A cDNA complemented the larval lethality of *rl* null alleles, as well as reversing the effects of a reduction in *rl* gene dosage during eye development. Our findings are consistent with the report that antisense MAP kinase blocks the proliferation of fibroblasts in response to fibroblast growth factor (Pages *et al.*, 1993). Recent studies provide evidence that MAP kinase is also required for RTK signaling during vulval induction in *Caenorhabditis elegans* (Lackner *et al.*, 1994; Wu and Han, 1994).

Although only a single MAP kinase has been identified in *Drosophila*, most species express multiple isoforms, suggesting that functional redundancy may be a characteristic of the MAP kinase family. The *Saccharomyces cerevisiae* MAP kinases, FUS3 and KSS1, are components of the signaling pathway activated by the binding of a/α mating pheromones to G-protein linked receptors (STE2 and STE3; Sprague and Thorner, 1992). Evidence for at least partial

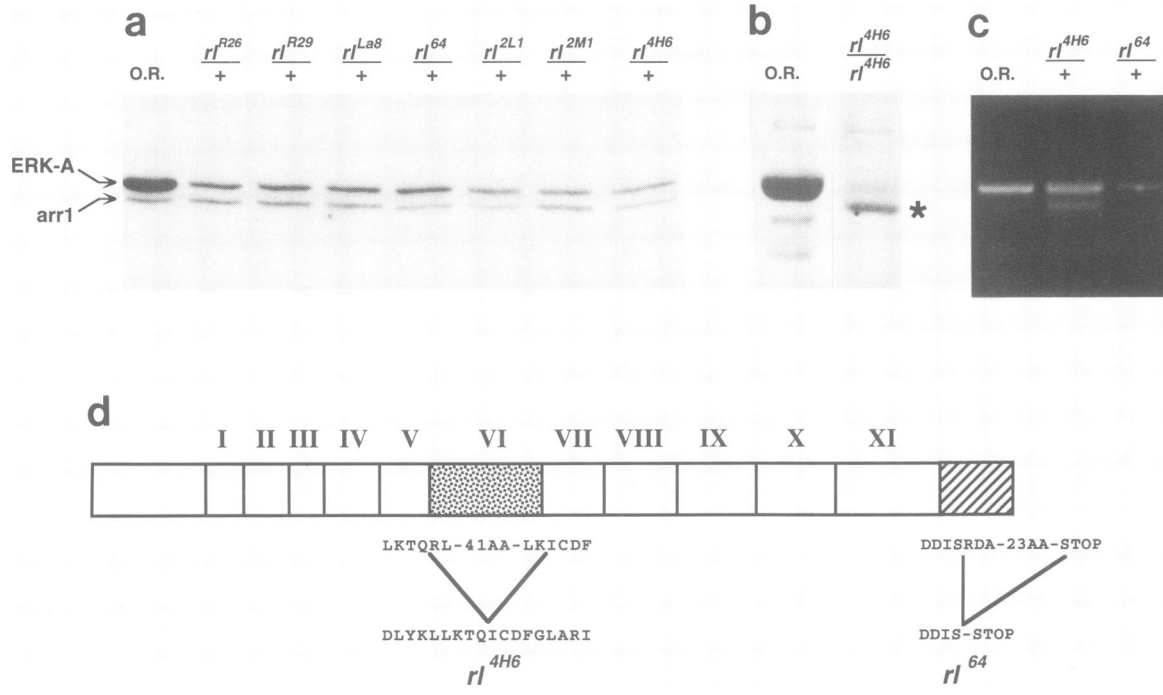

Fig. 3. Biochemical and molecular analyses of ERK-A in *rl* mutants. (**a**) Protein immunoblotting of ERK-A in head extracts prepared from wild-type (OR) flies and flies heterozygous for two X-ray (rl^{R26} and rl^{R29}) and five EMS-induced *rl* alleles (rl^{la8}, rl^{64}, rl^{2L1}, rl^{2M1} and rl^{4H6}). Levels of immunoreactivity were compared with Arrestin 1 (Dolph *et al.*, 1993) and quantified by phosphorimaging. For all *rl* alleles tested, there was an approximate two times reduction in ERK-A immunoreactivity (see Materials and methods). (**b**) Protein immunoblotting of larval extracts prepared from wild-type (OR) and mutant (rl^{4H6}/rl^{4H6}) third instar larvae. Asterisk (*) indicates position of mutant protein. (**c**) Ethidium bromide-stained agarose gel of RT-PCR products isolated from wild-type (OR) and heterozygous mutants ($rl^{4H6}/+$, $rl^{64}/+$). The upper band present in all three lanes corresponds in length to the wild-type ERK-A amplification product. The lower band present only in the $rl^{4H6}/+$ lanes corresponds to the truncated (135 bp) ERK-A amplification product. (**d**) Predicted effects on the ERK-A protein structure of mutations found in the ERK-A cDNA from rl^{64} and rl^{4H6} mutants. Protein kinase subdomains (I–XI) are indicated (Hanks *et al.*, 1988). The speckled box denotes subdomain VI, the region of ERK-A which is deleted in the rl^{4H6} mutation. The striped box indicates the region of ERK-A that is deleted in the rl^{64} mutation. The predicted amino acid sequences of the ERK-A wild-type and mutant proteins in these regions are also shown.

functional redundancy between FUS3 and KSS1 comes from the analysis of *fus3* and *kss1* mutants (Courchesne *et al.*, 1989; Elion *et al.*, 1990). The induction of mating-specific genes is reduced in either *kss1* or *fus3* mutants. In addition, *fus3* mutants also fail to arrest cell cycle progression in response to mating factor. However, while cells carrying either *fus3* or *kss1* mutations are capable of mating, *fus3;kss1* double mutants are sterile. These results demonstrate that FUS3 and KSS1 have overlapping, but non-identical, functions. The recent identification of two closely related MAP kinases in *C.elegans* may also provide a genetic system for assessing the functional redundancy between MAP kinases in RTK signal transduction pathways (Lackner, *et al.*, 1994).

Targets of rl/MAP kinase activity

What are the potential targets of *rl*-encoded MAP kinase (hitherto referred to as *rl*/MAP kinase) in the R7 precursor cell? One candidate is the nuclear protein encoded by the *seven in absentia* (*sina*) gene (Carthew and Rubin, 1990). Although genetic epistasis experiments indicate that *sina* acts downstream of *rl*/MAP kinase during R7 induction (Brunner *et al.*, 1993), it is unlikely that Sina function is directly regulated by *rl*/MAP kinase as it contains no consensus MAP kinase phosphorylation sites (Gonzalez *et al.*, 1991; Clark-Lewis *et al.*, 1991). Alternatively, *rl*/MAP kinase could, in principle, directly regulate the *yan* gene product, a negative regulator of R7 development, and photoreceptor development more generally; loss of function mutations in *yan* lead to the formation of additional photoreceptor cells (including R7s) in a signal-independent fashion (Lai and Rubin, 1992). *Yan* encodes a putative transcription factor containing an *ets* DNA binding domain and 19 potential MAP kinase phosphorylation sites. Importantly, MAP kinases have been shown to play a direct role in modulating the activity of transcription factors in response to extracellular cues in vertebrates (Hill *et al.*, 1993; Marais *et al.*, 1993). However, in contrast to the MAP kinase activation of other transcription factors described previously, genetic evidence suggests that *rl*/MAP kinase phosphorylation would negatively regulate the activity of Yan.

Other components of the Sev signaling pathway also contain consensus sites for MAP kinase phosphorylation and hence are potential targets for regulation by *rl*/MAP kinase. Although MAP kinase has been shown to phosphorylate Raf *in vitro*, the role of this phosphorylation is, as yet, unresolved (Anderson *et al.*, 1991; Lee *et al.*, 1992). Recent experiments demonstrate that MAP kinase is also capable of phosphorylating Sos *in vitro* (K.H.Zavitz, W.H.Biggs and S.L.Zipursky, unpublished observation), as well as interacting with Sos *in vivo*, in a yeast two hybrid system (X.Dong, K.H.Zavitz and S.L.Zipursky, unpublished observation). Since biochemical and genetic experiments indicate that both Sos and Raf act upstream of *rl*/MAP kinase, it is interesting to speculate that one function of MAP

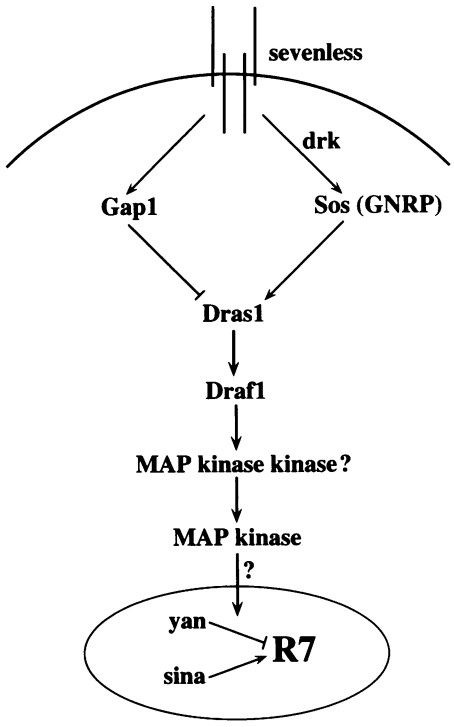

Fig. 4. The sevenless signal transduction pathway. The activation of the Sev RTK, present on the surface of the R7 precursor cell, occurs as a result of its interaction with the Boss ligand on the surface of the neighboring R8 cell (Krämer *et al.*, 1991; Hart *et al.*, 1993). The evidence for the involvement of other components and their order in the R7 inductive pathway are outlined below. For simplicity, it has been assumed that the components act in a linear, rather than in a parallel, manner. The formation of a complex including Sev, Drk (a SH2−SH3-containing protein) and Sos (a putative GNRP) has been demonstrated in co-immunoprecipitation experiments (Olivier *et al.*, 1993; Simon *et al.*, 1993). Although Gap1 (a putative GTPase activating protein) inhibits R7 development (Bonfini *et al.*, 1992; Buckles *et al.*, 1992; Gaul *et al.*, 1992), it is unclear if its activity is affected by the activation of Sev. Expression of *ras1*^{val12}, an activated form of Ras1, results in the *sev*-independent development of supernumerary R7 cells (Fortini *et al.*, 1992). Raf is required for the development of these extra R7 cells, placing it downstream of Ras1 (Dickson *et al.*, 1992b). *Dsor1*, a *Drosophila* homolog of MEK, has been shown to be required as part of the Torso signal transduction pathway downstream of Raf, but its function in the Sev pathway has not been demonstrated (Tsuda *et al.*, 1993). Evidence of a requirement for the *Drosophila* MAP kinase, ERK-A, in the R7 precursor is presented in this paper. Mutations in ERK-A suppress *raf*^{torY9}, a dominantly active form of Raf, placing it downstream of Raf. Loss-of-function alleles of *sina* suppress the gain-of-function allele of *rl*, *rl*^{Sem}, placing it downstream of ERK-A (Brunner *et al.*, 1994). Loss-of-function mutations in *yan* lead to *sev*-independent development of R7 cells that remain dependent upon *sina* (Lai and Rubin, 1992). This argues that *yan* acts upstream of *sina* to inhibit R7 development.

kinase may be in the feedback regulation of components required for its own activation.

rl/MAP kinase and specificity of cellular response

Genetic analysis reveals that *rl*/MAP kinase mediates diverse cellular responses downstream of the Sev, DER and Torso RTKs (Brunner *et al.*, 1994; and this paper). This raises the important issue of how the specificity of cellular response is controlled. The ability of a constitutively active form of the Torso RTK to drive R7 development when expressed in the developing retina suggests that the specificity of response lies downstream of the receptor (Dickson *et al.*, 1992a). One possibility is that cells of different developmental history may express different sets of pathway substrates. In the context of R7 development, two observations are consistent with this model. First, in the H214 enhancer trap line, β-galactosidase is expressed specifically in the R7 precursor cell independent of Sev function (Mlodzik *et al.*, 1992). Second, misexpression of the Rough homeobox protein in the R7 precursor cell drives this cell to assume a R1-6 cell fate in a Sev-dependent manner (Basler *et al.*, 1990; Kimmel *et al.*, 1990). Interestingly, the Rough protein contains a single consensus site for MAP kinase phosphorylation. This raises the possibility that the Sev-dependent phosphorylation of Rough by *rl*/MAP kinase may lead to the induction of genes specifying an R1-6 cell fate in the R7 precursor cell. Alternatively, activation of the Sev signal transduction pathway may simply be required to activate an R1-6 pathway of development previously specified by the misexpression of Rough.

In summary, this paper presents genetic, biochemical and molecular evidence that the *rl* locus encodes a *Drosophila* MAP kinase required in the Sev signal transduction pathway. The ability of *rl*^{Sem}, a gain of function allele of *rl*/MAP kinase, to drive R7 development independent of Sev indicates that activation of MAP kinase is not only necessary, but is sufficient for R7 induction (Brunner *et al.*, 1994). Genetic screens for enhancers and suppressors of loss and gain-of-function alleles of *rl*/MAP kinase may provide a way of identifying bona fide targets of MAP kinase, as well as an insight into the mechanisms by which MAP kinase activates a specific developmental pathway.

Materials and methods

Fly strains

The p[sE-*raf*^{torY9}] was carried on a CyO chromosome. *rl*^{1} is a weak loss-of-function allele of *rolled* (Morgan *et al.*, 1925). The *rl*^{la8}, *rl*^{64}, *rl*^{2L1}, *rl*^{2M1}, *rl*^{4H6}, *rl*^{R26} and *rl*^{R29} are complete loss-of-function alleles of *rolled*. The *rl*^{la8} and *rl*^{64} alleles were obtained from Dr Arthur Hilliker (University of Guelph, Ontario, Canada). The *rl*^{2L1}, *rl*^{2M1} and *rl*^{4H6} alleles were identified as dominant suppressors of sE-*raf*^{torY9} from a population of flies mutagenized with ethylmethane sulfonate (B.Dickson, A.van der Straten and E.Hafen, unpublished data). The *rl*^{R26} and *rl*^{R29} alleles were identified as X-ray-induced intragenic revertants of *rl*^{Sem}. The *w*^{1118}, *sev*^{E4};*Sos*^{JC2} stock was obtained from Dr Utpal Banerjee (UCLA, CA). The Oregon Red (OR) strain was used as wild-type in all of these studies.

Pseudopupil and scanning electron microscopy

Scanning electron microscopy and plastic sectioning were carried out as previously described (Kimmel *et al.*, 1990). The number of ommatidia containing R7 cells was determined using the corneal pseudopupil method (Franceschini and Kirshfeld, 1971; Banerjee *et al.*, 1987). The number of ommatidia scored is shown in parenthesis (Tables I and III). This represents the screening of eyes from 16 to 20 individuals.

Construct generation

2xsE-*ERK-A*^{WT} and 2xsE-*ERK-A*^{K164} transgenes were constructed in three steps. A unique *Bam*HI site was inserted immediately before the translation stop by the PCR method. A double-stranded oligonucleotide, with *Bgl*II 5′ overhangs, encoding the *myc* 9E10 epitope was inserted at this *Bam*HI site generating the *ERK-A*^{WT} clone (Evan *et al.*, 1985). This clone was sequenced to ensure that errors were not introduced during the PCR amplifications. The K164 mutation (Lys→Glu) was introduced into the WT *myc* clone by PCR. This mutation was first described as a dominant negative mutation in the *S.cerevisiae* MAP kinase homolog FUS3 (Ma *et al.*, 1993). *ERK-A*^{K164} *myc* clones were sequenced to ensure that errors were not introduced during the PCR. *ERK-A*^{WT} and *ERK-A*^{K164} were directionally cloned into the pKB267 transformation vector (Basler *et al.*, 1991). The pKB267 vector is a derivative of pW8 which contains a tandem repeat of the *sevenless* enhancer (6347−7564) (Basler *et al.*, 1989) as well as the

hsp70 promoter and transcriptional start (−250 to +90). Details of the construct generation are available upon request.

P element transformation

Drosophila germline transformation of the w^{1118} stock was carried out as previously described (Basler *et al.*, 1989). The p[2xsE-*ERK-A*^{WT}] and p[2xsE-*ERK-A*^{K164}] transformant lines used in these studies were homozygous viable insertions on the third chromosome, with no observable eye phenotype in a wild-type background (data not shown).

Heat shock rescue

Embryos were collected from a cross of rl^{4H6}/In(2LR)GlaElpBc;2xsE-*ERK-A*^{WT}/2xsE-*ERK-A*^{WT} and *Df(2R)*rl^{10a}/CyO and heat shocked throughout development; the heat shock regimen used was 40 min at 37°C followed by incubation for 7 h 20 min at 25°C. Embryos from crosses between rl^{4H6}/In(2LR)GlaElpBc;2xsE-*ERK-A*^{K164}/2xsE-*ERK-A*^{K164} and *Df(2R)*rl^{10a}/CyO were also heat shocked using the same paradigm. Embryos collected from a parallel series of crosses involving 2xsE-*ERK-A*^{WT} and 2xsE-*ERK-A*^{K164} were not subjected to the heat shock paradigm. *Df(2R)*rl^{10a} is a chromosomal deficiency, removing the *rl* as well as the closely linked *l(2)41Aa* lethal locus.

Reversion of rl/sE-raf^{torY9}

Flies with the genotype rl^{4H6}/CyO, p[sE-*raf*^{torY9}];2xsE-*ERK-A*^{WT}/+ were collected from a cross between rl^{4H6}/CyO, p[sE-*raf*^{torY9}] and rl^{4H6}/In(2LR)GlaElpB;2xsE-*ERK-A*^{WT}/2xsE-*ERK-A*^{WT}. Analogous crosses were set up with flies carrying 2xsE-*ERK-A*^{K164}. Scanning electron microscopy and plastic sectioning were carried out as previously described (Kimmel *et al.*, 1990).

Reversion of sev^{E4}*; Sos*^{JC2}*/rl*

Males were collected from a cross between *w,sev*^{E4}*;spd Sos*^{JC2}*/spd Sos*^{JC2};2xsE-*ERK-A*^{WT}/+ females and rl^{1}/rl^{1};2xsE-*ERK-A*^{WT}/2xsE-*ERK-A*^{WT} males. Males containing two copies of 2xsE-*ERK-A*^{WT} could be distinguished from their sibs with only one copy by virtue of their darker eye color. Males from an analogous cross with the 2xsE-*ERK-A*^{K164} transgene were also analyzed. The number of ommatidia containing R7 cells was determined using the corneal pseudopupil method (Franceschini and Kirshfeld, 1971; Banerjee *et al.*, 1987).

Protein immunoblotting

Extracts prepared from fly heads. Head extracts were prepared from wild-type (OR), two X-ray (rl^{R26} and rl^{R29}) and five EMS-induced *rl* alleles (rl^{la8}, rl^{64}, rl^{2L1}, rl^{2M1} and rl^{4H6}) as previously described (Biggs and Zipursky, 1992). Serial dilutions equivalent to 1.0, 0.5, 0.25 and 0.125 head were fractionated on an 8% SDS−polyacrylamide gel and immunoblotted as previously described (Biggs and Zipursky, 1992). The blots were probed with affinity-purified polyclonal antibodies against both ERK-A (732-2, 1:4000) and *Drosophila* Arrestin 1 (Dolph *et al.*, 1993) (arr1, 1:1000). The ERK-A antisera is directed against a synthetic peptide derived from the C-terminal (residues 347−364) of ERK-A (Biggs and Zipursky, 1992). The ERK-A immunoreactive protein migrates at ~45 kDa while the arr1 band migrates at ~42 kDa. Arrestin 1 is expressed at levels similar to that of ERK-A in adult head, and thus it serves as an internal control for sample loading. To quantify ERK-A and arr1, protein A−^{125}I was used as previously described (Harlow and Lane, 1988). After washing, the radiolabeled blot was imaged and quantified using the Molecular Dynamics Series 400 PhosphorImager and ImageQuant software.

Extracts prepared from rl^{4H6} *homozygous larvae.* The rl^{4H6}/rl^{4H6} larvae were identified and analyzed as follows. A 4 h embryo collection was made from bottles containing rl^{4H6}/In(2LR)*GlaElpBc* adults. The embryos were allowed to develop for 120 h at 25°C, corresponding to the late third instar stage of development. At this time, the rl^{4H6}/rl^{4H6} larvae, distinguished from sibs by the lack of the dominant *Black cell* marker (*Bc*), were ~1/10 the size of their balanced sibs (data not shown). Single larvae were homogenized in 10 μl of 2 × SDS−PAGE loading buffer and processed for protein immunoblotting. The presence of ERK-A was determined as described above. Immunoreactive bands were visualized using the Amersham ECL chemiluminescence system as described previously (Biggs and Zipursky, 1992).

Cloning of ERK-A cDNA from rl mutants

Total poly(A)$^+$ mRNA was isolated from adult flies heterozygous for rl^{4H6} and rl^{64} using the PolyA-Tract 1000 system (Promega). RT-PCR of the coding region of ERK-A (+252 to +1383) was performed as described previously (Biggs and Zipursky, 1992). Amplification products from three independent RT-PCRs of each allele were resolved by agarose gel electrophoresis and visualized by ethidium bromide staining. Bands were isolated, cloned into pBluescript KS (Stratagene) and sequenced using Sequenase 2.0 (US Biochemical).

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