14-3-3 ϵ positively regulates Ras-mediated signaling in *Drosophila*

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We have isolated mutations in the gene encoding a *Drosophila* 14-3-3 ϵ protein as suppressors of the rough eye phenotype caused by the ectopic expression of RAS1^{V12}. Using a simple loss-of-function 14-3-3 ϵ mutation, we show that 14-3-3 ϵ acts to increase the efficiency of RAS1 signaling. The 14-3-3 ϵ protein appears to function in multiple RTK pathways, suggesting that it is a general component of RAS1 signaling cascade. Sequence analysis of three dominant-negative alleles defines two regions of 14-3-3 ϵ that participate in RAS1 signaling. We also present evidence that 14-3-3 ϵ and 14-3-3 ζ , two 14-3-3 protein family members, are partially redundant for RAS1 signaling in photoreceptor formation and in animal viability. Our genetic data suggest that 14-3-3 ϵ functions downstream of or parallel to RAF, but upstream of nuclear factors in RAS1 signaling.

[Key Words: *Drosophila* 14-3-3 ϵ ; eye development; RAS1 signaling]

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The activation of the small GTPase Ras is an essential step in transducing signals initiated by receptor tyrosine kinases (RTKs) that function in diverse cellular processes (for review, see Schlessinger 1993). Thus, identifying and characterizing regulators and effectors of Ras has been a subject of considerable research effort. Proteins that link RTKs and Ras have been identified in various systems. These include the Sos guanidine nucleotide exchange factor (Rogge et al. 1991; Simon et al. 1991), the GRB2/sem-5/drk adaptor protein (Clark et al. 1992; Lowenstein et al. 1992; Simon et al. 1993; Olivier et al. 1993), and the GTPase activating protein (GAP; Gaul et al. 1992; Boguski and McCormick 1993). It is thought that Sos forms a complex with activated RTK via the adaptor protein and elevates the level of the active (GTP-bound) form of Ras. GAP, a negative regulator of Ras, completes the cycle by stimulating the intrinsic GTPase activity of Ras, returning Ras to the inactive (GDP-bound) state.

We have studied the events that occur in response to Ras activation during the development of the *Drosophila* eye. The *Drosophila* compound eye is comprised of an orderly array of 800 unit eyes, or ommatidia. Each ommatidium contains eight photoreceptor neurons, R1–R8, as well as four lens-secreting cone cells and several other accessory cells. Ras1 is required for the development of all photoreceptors (Simon et al. 1991) and its role in the determination of photoreceptor R7 has been studied extensively (for review, see Simon 1994; Wassarman et al.

1995). RAS1 transduces the signal initiated by SEVEN-LESS (SEV), an RTK expressed in a subset of cells including the presumptive R7. In the absence of SEV/RAS1 signaling, the R7 precursor cell fails to initiate neuronal differentiation and instead adopts the fate of a non-neuronal cone cell. In contrast, ectopic activation of the SEV/RAS1 pathway results in the transformation of some of the four cone cell precursors into supernumerary R7 cells (for review, see Zipursky and Rubin 1994).

Mutations in *Drosophila* homologs of Raf (*Draf*; Dickson et al. 1992; Karim et al. 1996), mitogen-activated protein kinase (MAPK) kinase (MEK) (*dsor1*; Tsuda et al. 1993; Karim et al. 1996), and MAPK [*rolled* (*rl*); Biggs et al. 1994; Brunner et al. 1994a; Karim et al. 1996] alter the strength of the SEV/RAS1 signaling pathway, suggesting that these proteins act downstream of RAS1. This genetic analysis is consistent with data from other systems, suggesting that the role of this MAPK cassette (Raf, MEK, and MAPK) in Ras signaling is conserved. In addition, a novel kinase, kinase suppressor of RAS (KSR), and a protein phosphatase, PP2A, have also been shown to regulate the activity of the pathway (Therrien et al. 1995; Wassarman et al. 1996). Inside the nucleus, two Ets-domain transcription factors (YAN, Lai and Rubin 1992; Tei et al. 1992; and POINTED, Brunner et al. 1994b; O'Neill et al. 1994), as well as SINA (Carthew and Rubin 1990) and PHYLLOPOD (PHYL; Chang et al. 1995; Dickson et al. 1995), two factors whose mechanisms of action remain unknown, have been implicated in R7 cell fate determination.

We have isolated additional downstream components of the *Drosophila* RAS1 signaling pathway in a genetic screen designed to identify mutations that alter the signaling effectiveness of an activated allele of *Ras1*,

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Ras1^{V12}. Here we report the cloning and characterization of *SR3-9* (*Suppressor of Ras1 3-9*), a suppressor of the rough eye phenotype caused by *Ras1*^{V12} (Karim et al. 1996). Molecular analysis indicated that *SR3-9* encodes a *Drosophila* homolog of the ϵ isoform of the 14-3-3 protein. The 14-3-3 proteins were initially identified as abundant brain proteins (Moore and Perez 1968) and were subsequently shown to be a mixture of seven to eight isoforms thought to be encoded by separate genes (Ichimura et al. 1987; Isobe et al. 1991). 14-3-3 proteins have been implicated in many processes (for review, see Aitken 1995) and have been shown to bind to many phosphoserine-containing signaling molecules (for review, see Aitken 1995; Muslin et al. 1996), including Raf-1 kinase (Freed et al. 1994; Irie et al. 1994). Although the ability of 14-3-3 to bind to Raf has been clearly demonstrated, the role of 14-3-3 in Ras signaling remains unclear. In *Drosophila*, a 14-3-3 protein of the ζ isoform (Skoulakis and Davis 1996) was identified and shown to play a role in learning and memory. However, it is not known whether *Drosophila* 14-3-3 ζ participates in Ras signaling.

Here we provide genetic evidence that removal of 14-3-3 ϵ function with a loss-of-function allele impairs RAS1 signaling efficiency, indicating that 14-3-3 ϵ functions positively in RAS1-mediated signaling. 14-3-3 ϵ appears to function in multiple RTK pathways, suggesting that it is a general component of the RAS1 signaling cascade. Sequence analysis of three dominant-negative alleles defines two regions of 14-3-3 ϵ that participate in RAS1 signaling. We also show that the ϵ and ζ proteins are partially redundant for RAS1 signaling in photoreceptor formation and in animal viability. Our genetic data suggest that 14-3-3 ϵ functions downstream of RAF, consistent with its proposed role as a modulator of activity or specificity of Raf kinase (Morrison 1994), or as a scaffold for

the assembly of signaling complexes (Brasselman and McCormick 1995; Muslin et al. 1996).

Results

SR3-9 mutants were isolated as suppressors of activated Ras1

To identify genes that participate in transmitting Ras1 signal, we (Karim et al. 1996) conducted a genetic screen for modifiers (suppressors and enhancers) of the rough eye phenotype caused by the expression of a constitutively active form of RAS1 under the control of the *sev* regulatory sequences, *sev-Ras1*^{V12}. The *sev-Ras1*^{V12} transgene results in the transformation of some of the four cone cell precursors into supernumerary R7 cells and a variety of other defects that disrupt the regular array of ommatidia, causing the exterior of the eye to appear rough (Fig. 1B; Fortini et al. 1992). Flies bearing two copies of the *sev-Ras1*^{V12} construct have rougher eyes than those that contain only one copy, suggesting that the degree of eye roughness is dependent on the level of RAS1^{V12} protein (data not shown). Because there is a correlation between the degree of eye roughness and the strength of the Ras1 signaling pathway, a twofold reduction in the dose of a gene (i.e., by mutating one copy) acting downstream of *Ras1* might alter the signaling strength of RAS1 sufficiently to visibly modify the rough eye phenotype of flies carrying the *sev-Ras1*^{V12} transgene. Based on this premise, we screened ~850,000 mutagenized flies and isolated mutations in a number of genes that modify this phenotype (Karim et al. 1996).

In this report we describe the characterization of *SR3-9*, a suppressor gene defined by two ethylmethane sulfonate (EMS)-induced mutant alleles isolated in this screen (Karim et al. 1996). These *SR3-9* alleles act as strong

Figure 1. *SR3-9* alleles suppress both activated Ras1 and Raf kinase. Scanning electron micrographs of the adult eyes of wild type (A), *sev-Ras1*^{V12}/+ (B), *sev-Ras1*^{V12}/+; *SR3-9*^{G96}/+ (C), *sev-Ras1*^{V12}/+; *SR3-9*^{j2B10}/+ (D), *sev-Ras1*^{V12}/+; *SR3-9*^{j2B10}/+ (E), *sev-Ras1*^{V12}/+; *SR3-9*^{j2B10}/*SR3-9*^{j2B10} (F), *sE-raf*^{T04021}/+ (G), and *sE-raf*^{T04021}/+; *SR3-9*^{G96}/+ (H). Anterior is to the right.

dominant suppressors of *sev-Ras1^{V12}*; that is, the eyes of *SR3-9/+; sev-Ras1^{V12}* flies are less rough than those of *sev-Ras1^{V12}* flies (Fig. 1, cf. C and D with B). In addition to suppressing the rough eye phenotype caused by *sev-Ras1^{V12}*, *SR3-9* alleles also suppress the supernumerary R7 phenotype. In *sev-Ras1^{V12}* flies, the number of R7 cells per ommatidium is increased from the wild-type number of 1.0 to 1.8 ($n = 69$); this number is decreased to 1.1 ($n = 200$) and 1.2 ($n = 260$) in flies that are also heterozygous for *SR3-9⁶⁹⁶* and *SR3-9¹²⁵⁹*, respectively.

The suppression of the *sev-Ras1^{V12}* phenotype was meiotically mapped to 3-62.0, which corresponds to the cytogenetic location of 90E-F. A deletion, *Df(3R)P14*, which deletes 90C2-D1 to 91A1-2 (Detwiler and MacIntyre 1978) and completely removes one copy of the *SR3-9* locus exhibits no dominant interaction with *sev-Ras1^{V12}* (data not shown), suggesting that the two EMS-induced alleles, *SR3-9⁶⁹⁶* and *SR3-9¹²⁵⁹*, are not simple loss-of-function mutations. Consistent with this interpretation, the percentage of normal ommatidia in flies homozygous for *SR3-9⁶⁹⁶* (62.5%; $n = 120$) is lower than that in *SR3-9⁶⁹⁶/Df(3R)P14* flies (69.1%; $n = 178$).

Homozygotes for a loss-of-function mutation suppress activated Ras1

We identified a P-element insertion in the *SR3-9* gene by screening P-element insertions mapped near the 90E-F region from the Berkeley *Drosophila* Genome Project collection (Bier et al. 1989; Spradling et al. 1995) for those that failed to complement *SR3-9⁶⁹⁶* by rough eye. One stock, *l(3)j2B10*, with a single P element inserted in 90E6-7, was found to fail to complement *SR3-9⁶⁹⁶*. Flies trans-heterozygous for *l(3)j2B10* and *SR3-9⁶⁹⁶* have rough eyes and a low penetrance of missing photoreceptors in ommatidia (data not shown). This phenotype could be reverted by mobilization of the P element, indicating that the P insertion was responsible for the non-complementation. This insertion appears to be a strong loss-of-function allele, as the rough eye phenotype of *SR3-9⁶⁹⁶/l(3)j2B10* is similar to *SR3-9⁶⁹⁶/Df(3R)P14* (data not shown).

During the mobilization of the P element in *l(3)j2B10*, we noticed that the recessive lethal mutation on that chromosome was not associated with the P-element insertion. After removing the lethal mutation by recombination, we could readily isolate flies homozygous for the P-element insertion, suggesting that *SR3-9* is not required for viability. We will refer to the P-insertion allele as *SR3-9^{j2B10}*. Flies homozygous for *SR3-9^{j2B10}* have normal eyes but are sterile.

Because the *SR3-9* mutations isolated from the *sev-Ras1^{V12}* screen are not simple loss-of-function alleles, we asked whether *SR3-9^{j2B10}*, a loss-of-function allele caused by gene disruption (see below), would suppress the *sev-Ras1^{V12}* phenotype. Flies heterozygous for *SR3-9^{j2B10}* exhibit no modification of the *sev-Ras1^{V12}* rough eye phenotype (Fig. 1, cf. E and B); however, flies homozygous for *SR3-9^{j2B10}* show strong suppression (Fig. 1, cf. F and B; the number of R7 per ommatidia is 1.19,

$n = 254$). This observation indicates that *SR3-9* functions positively in the RAS1 signaling pathway and the *SR3-9* gene activity needs to be reduced below 50% to alter the *sev-Ras1^{V12}* phenotype.

The dominant modifier screen is designed to isolate genes when a two-fold reduction in dose can alter the pathway enough to visibly affect the *sev-Ras1^{V12}* phenotype. However, if a particular signaling protein is in vast excess, then a >50% reduction may be required to visibly alter the rough eye phenotype. In this case, only dominant-negative alleles, like *SR3-9⁶⁹⁶* and *SR3-9¹²⁵⁹*, would modify the rough eye sufficiently to be detected.

SR3-9 functions downstream of Raf kinase

To further define the role of *SR3-9* in the RAS1 signaling cascade, we examined the genetic interaction between *SR3-9* and various components of the RAS1 pathway. We first asked whether *SR3-9* interacts with *Draf*, which encodes the *Drosophila* homolog of Raf kinase (Ambrosio et al. 1989). Expression of a constitutively active DRAF under control of the *sev* enhancer (*sE-raf^{Tor4021}*) produces a phenotype similar to *sev-Ras1^{V12}*, consistent with the idea that Raf responds to activation of Ras (Fig. 1G; Dickson et al. 1992). *SR3-9⁶⁹⁶* dominantly suppresses the rough eye defect caused by the *sE-raf^{Tor4021}* construct (Fig. 1H), indicating that *SR3-9* acts downstream of or parallel to RAF.

We then asked whether *SR3-9* interacts with *yan* or *phyl*, which encode two downstream targets of the SEV/RAS1 signaling cascade. Mutations in *yan*, which encodes an ETS-domain containing transcriptional repressor, result in the formation of supernumerary R7 cells and a rough eye phenotype (Lai and Rubin 1992; Tei et al. 1992; O'Neill et al. 1994). Ectopic expression of PHYL (*sev-phyl*), an early response gene in the SEV pathway, also generates extra R7 cells and produces a rough eye (Chang et al. 1995; Dickson et al. 1995). Although strongly suppressing the rough eye phenotypes of activated RAS1 and RAF, *SR3-9⁶⁹⁶* did not exhibit any detectable dominant interaction with either *yan* or *sev-phyl* (data not shown). These data suggest that *SR3-9* acts upstream of these nuclear factors.

The rough eye phenotype of *sE-raf^{Tor4021}* has also been used to identify additional components in the pathway (Dickson et al. 1996). Because our mutants interact strongly with *sE-raf^{Tor4021}*, we suspected that additional *SR3-9* alleles might have been isolated in this screen for modifiers of activated Raf. One suppressor, *18A2* (also an EMS-induced allele), has a map position similar to *SR3-9* and exhibits genetic interactions with other components in the pathway similar to *SR3-9* alleles (Dickson et al. 1996). We show below that *18A2* and *SR3-9* are allelic.

SR3-9 encodes a Drosophila homolog of 14-3-3ε

To identify the gene corresponding to *SR3-9*, genomic DNA flanking the *SR3-9^{j2B10}* insertion was recovered and used to screen a *Drosophila* eye-antennal imaginal disc cDNA library. Multiple clones of cDNAs that con-

tain identical open reading frames of 780 bp, but differ in the length of their 3' untranslated regions, were isolated. A diagram of the structure of the largest transcript is shown in Figure 2A. The conceptually translated protein of 260 amino acids exhibits a high degree of sequence similarity (82% identity) to human 14-3-3 ϵ (Chong et al. 1996; Fig. 2B). Although Dm14-3-3 ϵ and Dm14-3-3 ζ (Swanson and Ganguly 1992; Skoulakis and Davis 1996) share 82% and 81% identity with their respective human counterparts (Chong et al. 1996; GenBank accession no. U28964, G.A. Seluja, S.F. Pietromonaco, and L. Elias 1995), they share only 61% identity with each other (Fig. 2B), indicating that the difference between 14-3-3 proteins have been conserved in evolution.

Molecular analysis of the mutants indicates that *Drosophila melanogaster* 14-3-3 ϵ (Dm14-3-3 ϵ) corresponds to SR3-9. The P element in SR3-9^{j2B10} is inserted in the first intron of Dm14-3-3 ϵ locus (Fig. 2A). Furthermore, all three EMS-induced alleles from the modifier screens contain mis-sense mutations in the coding region of Dm14-3-3 ϵ (Fig. 2B): in SR3-9⁶⁹⁶, the glutamic acid at position 183 is changed into a lysine (E183K). In SR3-

9¹²⁵⁹, the phenylalanine at position 199 is changed into a tyrosine (F199Y). In 18A2, the tyrosine at position 214 is changed into a phenylalanine (Y214F). All three affected residues are conserved in other 14-3-3 proteins (Fig. 2B). We will refer to the SR3-9 locus as Dm14-3-3 ϵ , and these mutants as 14-3-3 ϵ ^{j2B10}, 14-3-3 ϵ ^{E183K}, 14-3-3 ϵ ^{F199Y}, and 14-3-3 ϵ ^{Y214F}.

To gain insight as to how these mutations might affect 14-3-3 function, we examined the location of affected residues in 14-3-3 structure. The structure of the 14-3-3 ϵ protein has not been solved. But because the primary sequences of the 14-3-3 protein family are well conserved, we reasoned that it would be appropriate to extend the analysis of the crystal structure of one 14-3-3 protein to another. In support of this assumption, the determined structures of human 14-3-3 τ and 14-3-3 ζ exhibit a high degree of similarity (Liu et al. 1995; Xiao et al. 1995). In Figure 2C, a ribbon diagram of the crystal structure of a dimer of human 14-3-3 ζ is shown (Liu et al. 1995). In the figure, the E183 (E180 in human 14-3-3 ζ) is positioned inside the pocket, with its side chain pointing toward the middle of the cavity. This pocket has been

Figure 2. SR3-9 encodes a *Drosophila* homolog of 14-3-3 ϵ isoform. (A) Map of the 14-3-3 ϵ locus at 90E6-7. The map is shown for the following restriction enzymes: EcoRI (R); XbaI (X); and BamHI (B). The horizontal line represents the genomic DNA (GenBank accession no. U84898). Below the line, the 14-3-3 ϵ cDNA (GenBank accession no. U84897) and the direction of the transcription are indicated. The solid boxes represent 14-3-3 ϵ coding regions; the open boxes represent untranslated regions. The $\blacktriangle$ in the 3'-untranslated region indicates an alternative site of 3'-end junction utilized in a 1.2-kb class of transcripts. The P element in SR3-9^{j2B10} (represented by a $\blacktriangle$) is inserted in the first intron (indicated by a line), ~1.8 kb after the first exon. The centromere is to the left. (B) The protein sequence alignment of Dm14-3-3 ϵ with human 14-3-3 ϵ (GenBank accession no. U54778; Chong et al. 1996), *Drosophila* 14-3-3 ζ (GenBank accession no. M77518; Swanson and Ganguly 1992; Skoulakis and Davis 1996), and human 14-3-3 ζ (GenBank accession no. U28964; G.A. Seluja, S.F. Pietromonaco, and L. Elias 1995). Amino acids identical to those of Dm14-3-3 ϵ are highlighted. The residues that are affected by the mutations isolated from the modifier screens are indicated by asterisks (see text for details). In 14-3-3 ϵ ⁶⁹⁶, the glutamic acid at position 183 is changed to a lysine (E183K); in 14-3-3 ϵ ¹²⁵⁹, the phenylalanine at position 199 is changed to a tyrosine (F199Y); in 14-3-3 ϵ ^{18A2}, the tyrosine at position 214 is changed to a phenylalanine (Y214F). (C) A ribbon diagram of human 14-3-3 ζ (Liu et al. 1995). The affected residues from B are indicated.

proposed as the site for substrate binding (Liu et al. 1995; Xiao et al. 1995), suggesting that the E183K change might affect this process. The F199 and Y214 (F196 and Y211 in human 14-3-3 ζ , respectively) are located next to each other, on a hydrophobic patch outside the pocket (Liu et al. 1995), suggesting that the EMS-induced 14-3-3 ϵ alleles can be classified into two groups based on their location.

The genetic properties of these mutations correlate with their location. 14-3-3 ϵ^{E183K} , the mutation in the putative substrate binding pocket, is the strongest suppressor of the three and is subviable. Rare homozygous escapers have rough eyes, with 37% of the ommatidia ($n = 120$) having missing photoreceptors. In contrast, flies homozygous for 14-3-3 ϵ^{F199Y} and 14-3-3 ϵ^{Y214F} , the mutations in the hydrophobic patch outside the pocket, are viable and fertile with no detectable phenotype except in sensitized backgrounds (i.e., *sev-Ras1^{V12}* and *sE-raf^{Tor4021}*). Together, these observations argue that these mis-sense mutations are affecting distinct domains of the 14-3-3 protein.

Mutations in 14-3-3 ϵ affect other RAS1-mediated pathways

It is becoming apparent that different RTKs utilize a similar set of proteins to transduce signals. Several lines of evidence suggest that 14-3-3 ϵ is also a general component in RAS1-mediated signaling pathways. All 14-3-3 ϵ mutants exhibit a dominant synthetic lethality with *Draf^{HIM7}*, a partial loss-of-function *Draf* allele that is viable at 18°C (data not shown; Melnick et al. 1993; Karim et al. 1996). Because the determination of R7 fate is completely dispensable for viability, this interaction with *Draf^{HIM7}* argues that 14-3-3 ϵ functions in other Ras1-dependent processes that are required for viability. Furthermore, flies homozygous for 14-3-3 ϵ^{E183K} often contain gaps in the posterior cross-veins of their wings (data not shown). This defect appears to be complementary to the phenotype caused by the removal of GAP1, where extra wing veins are formed in the middle of the cross-veins as a result of deregulated RAS1 activation (Gaul et al. 1992). This suggests, again, that 14-3-3 ϵ functions in other RAS1-mediated processes in addition to photoreceptor fate determination. It is also likely that 14-3-3 ϵ has other functions in development in addition to Ras1 signaling. Embryos derived from females homozygous for loss-of-function 14-3-3 ϵ mutations are arrested earlier (data not shown) than those derived from females homozygous for *Ras1* (Hou et al. 1995).

Functional redundancy among different 14-3-3 proteins

Because the eye phenotype of homozygous 14-3-3 ϵ mutants is significantly weaker than those of other components in the RAS1 pathway, we suspected that 14-3-3 ϵ and 14-3-3 ζ , the two known 14-3-3 proteins in *Drosophila*, might be functionally redundant in RAS1-mediated processes. As a first test of this hypothesis, we asked

Figure 3. Distinct 14-3-3 isoforms are functionally redundant in Ras1 signaling. Scanning electron micrographs of the adult eyes of 14-3-3 $\epsilon^{j2B10}/14-3-3\epsilon^{j2B10}$ (A) and 14-3-3 $\zeta^{X.1}/+$; 14-3-3 $\epsilon^{j2B10}/14-3-3\epsilon^{j2B10}$ (B). Anterior is to the right. Tangential sections of the adult retinæ of 14-3-3 $\epsilon^{j2B10}/14-3-3\epsilon^{j2B10}$ (C) and 14-3-3 $\zeta^{X.1}/+$; 14-3-3 $\epsilon^{j2B10}/14-3-3\epsilon^{j2B10}$ (D). The ommatidial composition of 14-3-3 $\epsilon^{j2B10}/14-3-3\epsilon^{j2B10}$ is basically wild type. Some ommatidia in 14-3-3 $\zeta^{X.1}/+$; 14-3-3 $\epsilon^{j2B10}/14-3-3\epsilon^{j2B10}$ (indicated by arrowheads) are missing either inner or outer photoreceptors.

whether mutations in the gene encoding 14-3-3 ζ [*leonardo* (*leo*) Skoulakis and Davis 1996] could modify the *sev-Ras1^{V12}* phenotype. Reduction of 14-3-3 ζ gene dose, in either flies heterozygous for *leo^{P2355}*, a lethal allele, or in flies homozygous for either of two viable alleles, *leo^{X.1}* and *leo^{2.3}*, did not detectably modify the *sev-Ras1^{V12}* phenotype (data not shown). However, reduction of the 14-3-3 ζ gene dose appears to worsen the phenotype of 14-3-3 ϵ mutants. Flies homozygous for 14-3-3 ϵ^{j2B10} are viable and have no apparent eye phenotype (Fig. 3A,C). However, mutating one copy of 14-3-3 ζ with *leo^{P2355}* in flies homozygous for 14-3-3 ϵ^{j2B10} results in lethality. Mutating one copy of 14-3-3 ζ with weaker alleles, *leo^{X.1}* or *leo^{2.3}*, in homozygous 14-3-3 ϵ^{j2B10} background results in a slight roughening of the eyes and a low penetrance (11%, $n = 146$) of missing photoreceptors (Fig. 3B,D). In addition to the abnormalities in the eye, the posterior cross-veins of >50% of these animals contain a gap. These observations suggest that different 14-3-3 proteins are partially redundant.

Discussion

We have isolated dominant-negative mutants in *Dm14-3-3 ϵ* as modifiers of activated RAS1. The fact that we isolated 14-3-3 ϵ mutations in an unbiased genetic screen, in which 14-3-3 ϵ and its putative targets are expressed at normal levels, provides strong evidence for the role of

14-3-3 ϵ in the Ras signaling pathway. We show that 14-3-3 ϵ plays a positive role as evidenced by the observation that reduction of 14-3-3 ϵ protein level by a loss-of-function allele impairs the strength of Ras1 signaling. We also show that the 14-3-3 ϵ and 14-3-3 ζ proteins are partially redundant.

The reports on the role of the 14-3-3 protein in Ras signaling in other systems appear to be conflicting. In the maturation of *Xenopus* oocytes and in certain tissue culture cells, overexpression of the 14-3-3 protein appears to elevate the efficiency of Ras signaling (Fantl et al. 1994; Freed et al. 1994; Li et al. 1995), suggesting that 14-3-3 has a positive role. On the other hand, dissociation of the 14-3-3 protein from the regulatory domain of Raf appears to render the Raf kinase constitutively active, suggesting that 14-3-3 has an inhibitory effect (Michaud et al. 1995; Suen et al. 1995; Rommel et al. 1996). Our data are most consistent with a positive function for 14-3-3. However, we cannot rule out the possibility that 14-3-3 proteins are used at distinct steps in the Ras signaling pathway but that only some of these steps are measured by our assays.

How do 14-3-3 ϵ proteins affect RAS1 signaling? Mammalian 14-3-3 proteins are known to bind to Raf kinase, suggesting that Raf activity could be a target of 14-3-3 regulation (Freed et al. 1994; Irie et al. 1994; Morrison 1994). The ability of 14-3-3 proteins to bind to many kinases raises the possibility that they might serve to coordinate the assembly of kinase complexes (Brasselman and McCormick 1995; Muslin et al. 1996). Our results provide genetic data in support of a positive role for Dm14-3-3 ϵ downstream of RAF in RAS1 signaling. Although membrane recruitment of Raf appears to be an important step in Raf activation (Leevers et al. 1994; Stokoe et al. 1994), 14-3-3 ϵ is probably not involved in this process, as we showed that 14-3-3 ϵ mutants suppress *sE-raf^{Tor4021}*, which expresses an activated RAF kinase that is already membrane bound (Dickson et al. 1992).

It seems clear that the alleles from the modifier screens are not loss-of-function mutations, as a 50% reduction of 14-3-3 ϵ gene dosage has no effect on *sev-Ras1^{V12}*. The simplest explanation for the genetic properties of these alleles is that they are dominant negatives. 14-3-3 proteins are thought to function as dimers (Liu et al. 1995; Xiao et al. 1995); therefore, these mutants could titrate out the wild-type pool of 14-3-3 ϵ proteins by forming nonfunctional dimers. Furthermore, because 14-3-3 proteins are able to bind to many signaling molecules (for review, see Aitken 1995), these nonfunctional dimers might titrate out factors that are essential for transducing signals. One could imagine that if the role of 14-3-3 proteins is to act as a scaffold for assembling signal complexes, the presence of a mutant 14-3-3 monomer incapable of binding a particular complex component, or binding too tightly, could render the whole complex nonfunctional.

Loss of 14-3-3 ϵ function has only mild effect on viability and eye development, yet removing only one copy of 14-3-3 ϵ by a loss-of-function mutation enhances the lethality of a weak loss-of-function *Draf* allele. It is pos-

sible that 14-3-3 ϵ is not required for RAS1 signaling but, rather, modulates the efficiency of signaling, and in the absence of 14-3-3 ϵ function, sufficient RAS1 signaling still can occur. Only when the system is made sensitive by mutations of other components are phenotypes due to loss of 14-3-3 ϵ observed. Alternatively, if different 14-3-3 family members can substitute for one another during RAS1 signaling, then removal of only one isoform might not be expected to produce a strong phenotype. A recent analysis of the *Drosophila* 14-3-3 ζ protein by Kockel et al. (1997) suggests that it plays a role in photoreceptor formation during eye development. Our observation that reduction of 14-3-3 ζ gene dosage enhances the phenotype of 14-3-3 ϵ indicates that there is some overlap in the function of different 14-3-3 proteins in photoreceptor development and viability. Still, the remarkable conservation of individual isoforms in evolution argues strongly that they have distinct functions in cellular physiology (for review, see Aitken et al. 1992).

The position of the EMS-induced mutations in the 14-3-3 structure suggests that 14-3-3 proteins have at least two regions that play a role in modulating Ras signaling. One is within the putative binding pocket. However, the other domain has not been implicated previously in any process. The subtle molecular nature of the mutations of this domain, phenylalanine to tyrosine and tyrosine to phenylalanine, is striking given the strong dominant-negative phenotype produced by these mutant proteins in the *sev-Ras1^{V12}* background. These two regions could cooperate at one step or act in different steps during Ras signaling. Identification of molecules that interact with these two domains should enhance our understanding of the role of the 14-3-3 protein.

Materials and methods

Genetics

Fly culture and crosses were performed according to standard procedures. The screen for dominant modifiers of *sev-Ras^{V12}* is described in detail by Karim et al. (1996). Flies carrying the *raf^{HM7}* allele were raised at 18°C; all other crosses were performed at 25°C. The *sev-Ras1^{V12}*, *sE-raf^{Tor4021}*, *yan*, and *sev-*phyl** lines used for interaction assay were *Cyo*, *sev-Ras1^{V12}-2* (*CR2*), *BT98*, *yan¹*, and *sev-*phyl*-1*, respectively. *Df(3R)P14* was obtained from the Bloomington Stock Center.

Histology

Scanning electron microscopy was performed as described by Kimmel et al. (1990). Fixation and sectioning of adult eyes were performed as described by Tomlinson and Ready (1987). Cuticle preparation of embryos was performed as described in Belvin et al. (1995).

DNA sequencing

DNA sequence was determined by the chain termination method (Sanger et al. 1977) using the Automated Laser Fluorescence system (A.L.F., Pharmacia). Templates were prepared by sonication of plasmid DNA and insertion of sonicated DNA into M13mp10 vector. DNA from two cDNA clones, as well as most of the 9- and 1.2-kb genomic *EcoRI* DNA fragments, were

sequenced completely on both strands. The DNA sequences were processed and analyzed by Staden (Roger Staden, MRC Laboratory of Molecular Biology, Cambridge, UK) and UWGCG (University of Wisconsin) software packages.

To demonstrate that *SR3-9* mutants contain lesions in *Dm14-3-3 ϵ* transcript, genomic DNA that contains the exons of the *14-3-3 ϵ* locus were isolated from wild-type and homozygous mutant flies. The genomic region corresponding to the *14-3-3 ϵ* coding sequence was PCR amplified and the PCR products were then used for sequence analysis. Mutations were determined by comparison to the *w; iso 2; 3* parental DNA sequence for this region. Multiple independent PCR products were analyzed to exclude the possibility of PCR-induced errors.

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