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The heat shock protein 83 (Hsp83) is required for Raf-mediated signalling in *Drosophila*

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The heat shock protein Hsp90 has been shown to associate with various cellular signalling proteins such as steroid hormone receptors, src-like kinases and the serine/threonine kinase Raf. While the interaction between steroid hormone receptors and Hsp90 appears to be essential for ligand binding and activation of the receptors, the role of Hsp90 in Raf activation is less clear. We have identified mutations in the *hsp83* gene, the *Drosophila* homologue of *hsp90*, in a search for dominant mutations that attenuate signalling from Raf in the developing eye. The mutations result in single amino acid substitutions in the Hsp83 protein and cause a dominant-negative effect on the function of the wild-type protein. We show that both wild-type and mutant forms of Hsp83 bind to the activated *Drosophila* Raf but the mutant Hsp83 protein causes a reduction in the kinase activity of Raf. Our results indicate that Hsp83 is essential for Raf function *in vivo*.

Keywords: chaperone/Hsp90/Raf/Sevenless/signal transduction

Introduction

Subjecting any living cell to a heat shock results in the rapid induction of a highly conserved group of proteins, the heat shock proteins (for review, see Parsell and Lindquist, 1993). Many of these proteins are required not only for stress tolerance, but also at normal physiological temperatures for processes such as protein folding and oligomer assembly. One family of heat shock proteins, the Hsp90 family, has also been implicated as an important component of intracellular signalling pathways. Dimeric Hsp90 proteins bind molecules such as steroid hormone receptors (Catelli *et al.*, 1985) and the kinases v-src, Raf and casein kinase II (Miyata and Yahara, 1992; Stancato *et al.*, 1993; Xu and Lindquist, 1993; Wartmann and Davis, 1994). In the case of steroid receptors, this interaction is required for efficient ligand binding and transcriptional regulation (Bohen and Yamamoto, 1993). Here we present genetic and biochemical data suggesting that Hsp90 also plays an important role in signalling via the Raf serine/threonine kinase.

En route to the nucleus, many signals originating extracellularly pass through the Raf kinase. One well characterized pathway begins with the activation of a receptor tyrosine kinase (RTK) in response to some external stimulus. RTK activation in turn leads to Ras activation, via intermediates such as Grb2, Sos, Dos, Shc and Gab1 (Holgado-Madruga *et al.*, 1996; Raabe *et al.*, 1996; for review, see Pawson, 1995). Activated Ras then recruits Raf to the plasma membrane and stimulates its kinase activity (Leevers *et al.*, 1994; Stokoe *et al.*, 1994). Raf acts at the head of a serine/threonine kinase cascade that ultimately leads to the phosphorylation and nuclear translocation of MAPK (for review, see Marshall, 1994). Once in the nucleus, MAPK is able to phosphorylate a number of transcription factors, and thus alter the pattern of gene expression (for review, see Dickson, 1995).

The mechanism by which Raf is activated is not well understood. In mammalian cells, it has been shown that recruitment of Raf to the membrane by binding to Ras cannot account for full activation of Raf. A membrane-anchored form of Raf can be stimulated further by epidermal growth factor (EGF) treatment in a Ras-independent manner (Leevers *et al.*, 1994), and tyrosine phosphorylation by src-like kinases has been shown to potentiate Raf activity (Marais *et al.*, 1995). Furthermore, in addition to Hsp90, members of the 14-3-3 family of proteins are also known to be constitutively associated with Raf (for review, see Aitken, 1995). Little is known, however, about the role of these proteins in Raf activation.

A powerful model system for the analysis of Raf signalling *in vivo* is the specification of the R7 photoreceptor in the developing eye of *Drosophila melanogaster* (for reviews, see Zipursky and Rubin, 1994; Domínguez and Hafen, 1996). In this case, the external stimulus is the protein Boss, expressed on the surface of the neighbouring R8 cell. Boss is the ligand for the Sevenless (Sev) RTK, expressed by several undetermined cells in the developing eye imaginal disc. Some of these cells, the precursors of the R7 photoreceptor and the four non-neuronal cone cells, together form the R7 equivalence group, since each has the potential to become either an R7 cell or a cone cell, depending on whether or not the Sev RTK is activated (Greenwald and Rubin, 1992). The presumptive R7 cell is the only member of this group that makes direct contact with the R8 cell, and thus is the only cell in which Sev can be activated by its ligand Boss. This spatial restriction can be overcome either by ectopic expression of Boss or by direct activation of Sev in the cone cell precursors (Basler *et al.*, 1991; Van Vactor *et al.*, 1991). In either case, the precursors of cone cells are induced to differentiate as additional R7 cells.

Sev activates a signalling pathway that involves the adaptor proteins Drk (a Grb2 homologue) (Olivier *et al.*, 1993; Simon *et al.*, 1993) and Daughter-of-sevenless (Dos) (Herbst *et al.*, 1996; Raabe *et al.*, 1996), the guanine nucleotide release factor Son-of-sevenless (Sos) (Rogge *et al.*, 1991; Simon *et al.*, 1991), the Ras protein Ras1 (Simon *et al.*, 1991) and the kinases Ksr (Therrien *et al.*,

1995), Raf (Dickson *et al.*, 1992), MEK-1 (or Dsor1) (Tsuda *et al.*, 1993; Lu *et al.*, 1994) and the Rolled MAPK (Biggs *et al.*, 1994; Brunner *et al.*, 1994). Activation of this pathway at points downstream from Sev, such as Ras1, Raf or Rolled, bypasses the need for Sev activation to induce the R7 fate in all members of the R7 equivalence group (Dickson *et al.*, 1992; Fortini *et al.*, 1992; Brunner *et al.*, 1994). Also, as a general rule, a loss-of-function mutation in a gene encoding one of these signalling molecules impairs signalling from a constitutely activated form of an immediately upstream component, and thereby suppresses the ability of this activated molecule to induce the recruitment of additional R7 cells. For example, Ras1 mutations dominantly suppress the multiple R7 phenotype caused by constitutive activation of the upstream component Sev, but have no effect on constitutively activated downstream components such as Raf or Rolled.

With the aim of identifying other molecules involved in this signalling process, we recently performed a genetic screen for mutations that dominantly suppress the multiple R7 phenotype caused by constitutive activation of the Raf kinase (Dickson *et al.*, 1996). We show here that the strongest dominant suppressor mutations isolated in this screen disrupt the *hsp83* gene, which encodes the *Drosophila* Hsp90 protein. *hsp83* mutations had also been isolated previously on the basis of genetic interactions with a temperature-sensitive *sev* allele (Simon *et al.*, 1991; Cutforth and Rubin, 1994). We demonstrate here that Hsp83 physically associates with Raf and that mutant forms of Hsp83 cause a reduction in Raf kinase activity. These results demonstrate that Hsp83 protein is required for Raf function in *Drosophila*.

Results

Su(Raf)3A encodes a protein generally required for Raf signalling

We previously have reported the isolation of several mutations which dominantly suppress the formation of multiple R7 cells as a response to constitutive activation of the Raf kinase in all members of the R7 equivalence group (Dickson *et al.*, 1996). These mutations define seven *Su(Raf)* loci. The molecular characterization of two of these has already been reported. One of these is the gene *rolled*, which encodes a *Drosophila* MAP kinase (Biggs *et al.*, 1994; Brunner *et al.*, 1994), most likely a general component mediating signal transduction downstream of Raf. The second *Su(Raf)* loci to be cloned, *phyllopod* (*phyl*) (Chang *et al.*, 1995; Dickson *et al.*, 1995), appears to be a target gene of the Raf/MAPK pathway that is required in only a few specific responses to Raf signalling, including induction of the R7 cell fate, as well as that of the R1 and R6 photoreceptors. Mutations in either of these genes suppress the dominant rough eye phenotype of Raf^{torY9} flies (described in Dickson *et al.*, 1992), but differ markedly in their interactions with a hypomorphic *raf* allele, raf^{HM7}. Hemizygous raf^{HM7} flies show reduced viability, as well as the absence of the R7 cell in ~50% of the ommatidia. Both *rl* and *phyl* mutations dominantly enhance the raf^{HM7} phenotype, but whereas the raf^{HM7} allele becomes completely lethal in a *rl*/+ background, no reduction of viability is seen at all in a *phyl*/+ background. The interaction with raf^{HM7} thus provides a simple means of distinguishing components required generally for Raf function throughout development from those required specifically for Raf function in R7 specification.

On the basis of its genetic interactions with Raf^{torY9} and raf^{HM7}, a third *Su(Raf)* locus, *Su(Raf)3A*, presented itself as a strong candidate for another gene encoding a protein generally required for Raf signalling. The two *Su(Raf)3A* alleles, *9J1* and *13F3*, were the strongest dominant suppressors of the Raf^{torY9} phenotype we isolated, completely eliminating all additional R7 cells (Figure 1C and G, Figure 4A). The strong suppression is specific for Raf^{torY9}, since *9J1* and *13F3* do not strongly suppress the rough eye phenotype caused by the constitutive activation of Ras1 ($sev\text{-}Ras^{V12}$) or Rl/MAP kinase (rl^{DN}) (Figure 4E). Furthermore, as with *rl*, the raf^{HM7} mutation becomes lethal in a *Su(Raf)3A*/+ background, and it is not possible to recover homozygous *Su(Raf)3A* clones in adult flies (data not shown, and Simon *et al.*, 1991). We therefore decided to proceed with the molecular characterization of this locus.

Su(Raf)3A is the hsp83 gene

Mapping by meiotic recombination initially placed the *Su(Raf)3A* gene at position 13 ± 4 on the left arm of the third chromosome, and deficiency mapping localized the gene to the cytological interval 63A1; C1. In addition to the two ethyl methanesulfonate (EMS)-induced alleles recovered as suppressors of Raf^{torY9}, we also identified a lethal P element insertion, *P582* (P.Deak and P.Maröy, unpublished data), that failed to complement both EMS-induced alleles. This insertion was localized to 63B5-11 on polytene chromosomes, and precise excision by P element transposase demonstrated that this insertion was responsible for the lethality associated with this chromosome.

We cloned the region flanking the *P582* insertion (Figure 2A) and determined that this P element lies within the 5′-untranslated region (5′-UTR) of the *hsp83* gene (Hackett and Lis, 1983). A construct containing 7.5 kb of genomic DNA from this region, including both *hsp83* and an adjacent transcript (Wohlwill and Bonner, 1991), rescued the lethality of *Su(Raf)3A* mutations in transgenic flies. Furthermore, introducing this construct into a Raf^{torY9}/+; *Su(Raf)3A*/+ background resulted in the reappearance of additional R7 cells. To demonstrate that it was the *hsp83* gene and not the adjacent transcript (O'Connor and Lis, 1981) that was responsible for the abolition of suppression, we generated transgenic flies in which the *hsp83* cDNA was expressed under the control of the *sev* enhancer (Basler *et al.*, 1989), and thus in the cells of the R7 equivalence group. As with the genomic construct, this *sev–hsp83* construct also restored additional R7 cells in a Raf^{torY9}/+; *Su(Raf)3A*/+ background (Figure 1D and H).

Finally, we cloned and sequenced the *hsp83* gene from both $Su(Raf)3A^{9J1}$ and $Su(Raf)3A^{13F3}$ strains. Single point mutations were identified for each allele (Figure 2B), in regions that are highly conserved amongst the *Drosophila*, yeast and human Hsp90 family members. Since neither the *P582* insertion nor deficiencies that completely remove the *hsp83* gene act as dominant suppressors of the Raf^{torY9} phenotype, we infer that both EMS-induced mutations are antimorphic in nature.

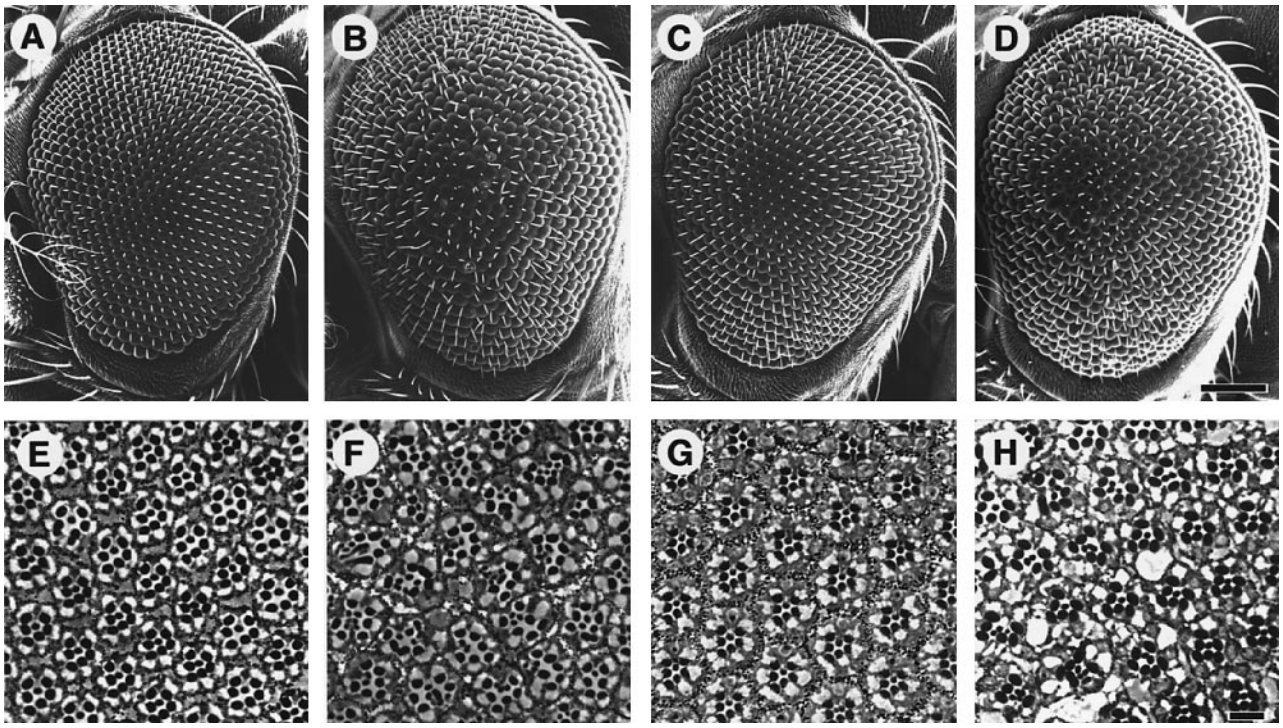

Fig. 1. The *Raf*^{torY9} gain-of-function phenotype is suppressed by *Su(Raf)3A*. Scanning electron micrographs (A–D) and histological sections (E–H) of eyes of flies of the following genotypes are shown: wild-type (**A** and **E**), *Raf*^{torY9}/+ (**B** and **F**), *Raf*^{torY9}/+; *Su(Raf)3A*^{9J1}/+ (**C** and **G**), *sE-hsp83*/+; *Su(Raf)3A*^{9J1}, *Raf*^{torY9}/+ (**D** and **H**). The *Raf*^{torY9} flies have rough eyes due to the recruitment of multiple R7 photoreceptor cells (F). Removal of one copy of *Su(Raf)3A* prevents this recruitment of additional R7 cells, reverting the Raf^{torY9} phenotype to almost wild-type (G). Addition of one wild-type copy of the *hsp83* gene rescues the suppression and restores the multiple R7 cells (H). The scale bars represent 100 μm in (D) and 10 μm in (H).

Five additional *hsp83* alleles have been isolated previously as dominant enhancers of a temperature-sensitive loss-of-function *sev* allele [*E(sev)*] (Simon *et al.*, 1991; Cutforth and Rubin, 1994). All five mutations also result in single amino acid substitutions. There is, however, no obvious correlation between the site of a mutation in the primary sequence and its recovery as either a *Su(Raf)* or *E(sev)* mutation (Figure 2B).

Intragenic complementation between hsp83 alleles

Surprisingly, *hsp83* alleles recovered as *E(sev)* mutations generally complement those recovered as *Su(Raf)* mutations, with the exception that two *E(sev)* alleles, *hsp83*^{e4A} and *hsp83*^{e6D}, do not complement the *Su(Raf)* allele *hsp83*^{13F3} (Figure 3). The viable heteroallelic combinations result in male sterility, but no other signs of abnormal development. The P element allele, *P582*, does not complement any of the EMS-induced alleles, and all alleles are lethal over a deficiency for the locus.

Since Hsp90 proteins act as dimers (Minami *et al.*, 1994), a likely explanation for the existence of these two classes of mutation is that they affect different functional domains of the protein, such that certain heteroallelic combinations result in functional heterodimers, even if the homodimers are not functional. The broad correlation between these two complementation groups and their recovery as *E(sev)* and *Su(Raf)* mutations suggested that the former mutations may specifically disrupt an interaction with Sev, the latter specifically an interaction with Raf. To verify this hypothesis, we tested the *E(sev)* alleles for interactions with Raf and the *Su(Raf)* alleles for interactions with Sev.

As shown in Figure 4, the *Su(Raf)* alleles also interact genetically with both gain- and loss-of-function *sev* alleles, though these interactions appear to be generally weaker than the interactions with *raf*. Conversely, four of the five *E(sev)* alleles showed no genetic interaction with either *raf* or *sev*. One of the five *E(sev)* alleles, *hsp83*^{e6D}, did, however, show strong interactions with both *sev* and *raf* alleles, acting as an antimorph. Since both *Su(Raf)* alleles, as well as the *E(sev)* allele *hsp83*^{e6D}, interact genetically with both *raf* and *sev*, we conclude that the two classes of mutations probably do not specifically disrupt domains required for interactions with *raf* or *sev*, respectively. Rather, the distinction appears to be one of strength: the *Su(Raf)* alleles are strongly antimorphic; most of the *E(sev)* alleles are not. Antimorphic alleles were recovered in the *Raf*^{torY9} screen at a frequency of 1/100 000, and it is therefore not surprising that only one such allele was recovered in the *sev*^{ts} screen, in which only 30 000 flies were screened. Conversely, hypomorphic *E(sev)* alleles, recovered at high frequency (1/6000) in the *sev*^{ts} screen, do not interact with *Raf*^{torY9} and thus could not have been recovered in the *Raf*^{torY9} screen.

Reconsidering the locations of the various *hsp83* mutations in this light, one can observe a weak correlation between the site of a mutation and its genetic nature: all but one of the hypomorphic alleles, and none of the antimorphs, map to the C-terminal domain known to be involved in dimerization (Minami *et al.*, 1994).

Wild-type and mutant Hsp83 proteins bind Raf

It has been reported that human c-Raf-1 and Hsp90 proteins form part of a large, multi-subunit complex

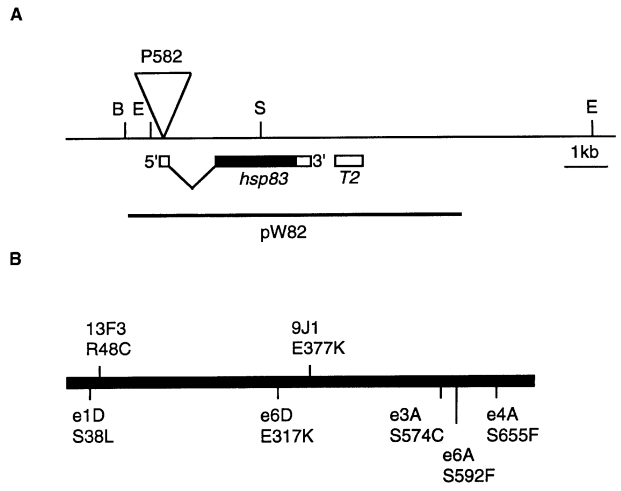

Fig. 2. *Su(Raf)3A* encodes the heat shock protein 83 (Hsp83). (**A**) A restriction map of 12 kb of the genomic region encompassing the *hsp83* gene is shown. The region was isolated by means of the plasmid rescue technique (Mlodzik *et al.*, 1990) using the P element insertion line *P582* (P.Deak and P.Maröy, unpublished results). The insertion point of the P element is located 29 bp downstream of the *hsp83* transcription start site. cDNAs derived from the *Hsp83* mRNA were isolated from an eye disc cDNA library (prepared by A.Cowman). The position of the flanking transcript T2 (0.8 kb) is approximate, since the genomic extent of this transcript has not been determined (O'Connor and Lis, 1981; Blackmann and Meselson, 1986). pW82 represents the genomic DNA clone (Wohlwill and Bonner, 1991) that rescues the suppression as well as the lethality of *Su(Raf)3A* mutants. The restriction sites for *Bam*HI (B), *Eco*RI (E) and *Sal*I (S) are indicated. (**B**) The coding region of *hsp83* is shown schematically as a horizontal bar. The positions and the resulting amino acid substitutions of the seven EMS-induced *hsp83* mutations are shown above the bar for the two *Su(Raf)* alleles and below the bar for the five alleles identified and characterized as *E(sev)* mutations by Cutforth and Rubin (1994). The positions of the amino acid exchanges are as follows: 9J1, E377K (G1129A); 13F3, R48C (C142T); e1D, S38L (C113T); e3A, S574C (C1721G); e4A, S655F (C1964T); e6A, S592F (C1775T); e6D, E317K (G949A).

(Stancato *et al.*, 1993; Wartmann and Davis, 1994). To test for possible physical interactions between the *Drosophila* Hsp83 protein and Raf, we co-transfected *Drosophila* Schneider cells with constructs encoding a c-myc epitope-tagged Hsp83 protein (Hp83-myc) and an activated form of Raf ($Raf^{tor4021}$). $Raf^{tor4021}$ is identical to Raf^{torY9} except that it contains a different amino acid subsitution in the Torso extracellular domain which results in a stronger activation of the Raf kinase *in vivo* (Dickson *et al.*, 1992). The use of a c-myc tag (Evan *et al.*, 1985) allowed us to distinguish the transfected and endogenous versions of the Hsp83 protein, so that subsequently we could test mutant forms of the protein. Cells co-expressing Hsp83-myc and $Raf^{tor4021}$ were lysed and immunoprecipitated with antibodies against Raf. Immunoprecipitates were separated by SDS–PAGE and analysed on Western blots using the monoclonal antibody 9E10 (BAbCO) against the c-myc tag. These blots showed that Hsp83 associates strongly with Raf (Figure 5).

We next tested mutant forms of the Hsp83 protein for binding to Raf. To do this, we introduced into the Hsp83-myc construct the point mutations identified in the *Su(Raf)* alleles $hsp83^{9J1}$ and $hsp83^{13F3}$, as well as two of the *E(sev)* alleles, $hsp83^{e1D}$ and $hsp83^{e6D}$. Co-immunoprecipitation assays showed that all four mutant proteins are still able to bind Raf, although compared with wild-type Hsp83 the two *E(sev)* mutant proteins showed somewhat reduced binding. We cannot exclude the possibility, however, that these mutant proteins bind to Raf as part of complexes with endogenous wild-type Hsp83.

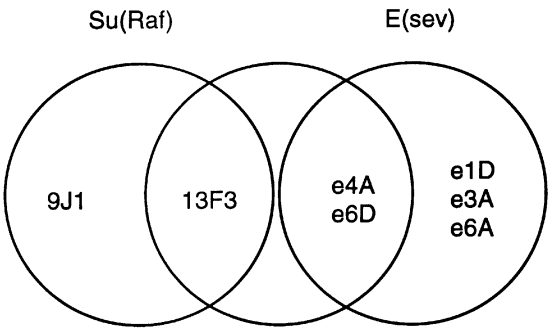

Fig. 3. Intragenic complementation of *hsp83* alleles. A Venn diagram indicating subsets of *hsp83* alleles that fail to complement each other. For example, *9J1* complements all other EMS-induced alleles except *13F3*, which in turn is lethal over *e4A* and *e6D* but viable over *e1D*, *e3A* and *e6D*. Note that two of the subsets of non-complementing *hsp83* alleles correspond to the *E(sev)* and *Su(Raf)* classes. The P element-induced allele *P582* fails to complement all EMS-induced alleles.

Hsp83 mutations reduce Raf kinase activity

Since mutant Hsp83 proteins are still able to bind the Raf protein, we next tested whether they cause a reduction in Raf^{torY9} kinase activity. Larvae heterozygous for Raf^{torY9} and either one of these four *hsp83* alleles were heat shocked for 1 h at 37°C to induce ubiquitous expression of the Raf^{torY9} transgene. Cell-free extracts were prepared and incubated with recombinant kinase-inactive GST–MEK. Upon heat induction, the Raf kinase activity in Raf^{torY9} larval extracts was increased. The presence of a single copy of the four *hsp83* alleles tested resulted in a marked reduction in Raf kinase activity (Figure 6). These experiments indicate that although the mutant Hsp83 proteins are still able to bind to Raf they weaken signalling from Raf^{torY9} by directly reducing Raf kinase activity.

Discussion

The heat shock proteins are induced rapidly at elevated temperatures, but are also expressed at high levels at normal growth temperatures (for review, see Parsell and Lindquist, 1993). One function of these proteins is to facilitate protein folding, and they are probably induced at higher temperatures to meet the increased demand for the refolding of denatured proteins. Another function of these proteins, which is probably not temperature dependent, appears to be to stabilize particular conformational states and facilitate the transition from one state to another. It is in this regard that they are likely to play a critical role in signal transduction pathways, in which accurate switching is required between the inactive and active states of various signalling molecules.

One of the best understood interactions between a heat shock protein and a signal transduction molecule is that between the Hsp90 protein and steroid receptors (for a review see Bohen and Yamamoto, 1994). In the absence of a ligand, many steroid receptors have been shown to

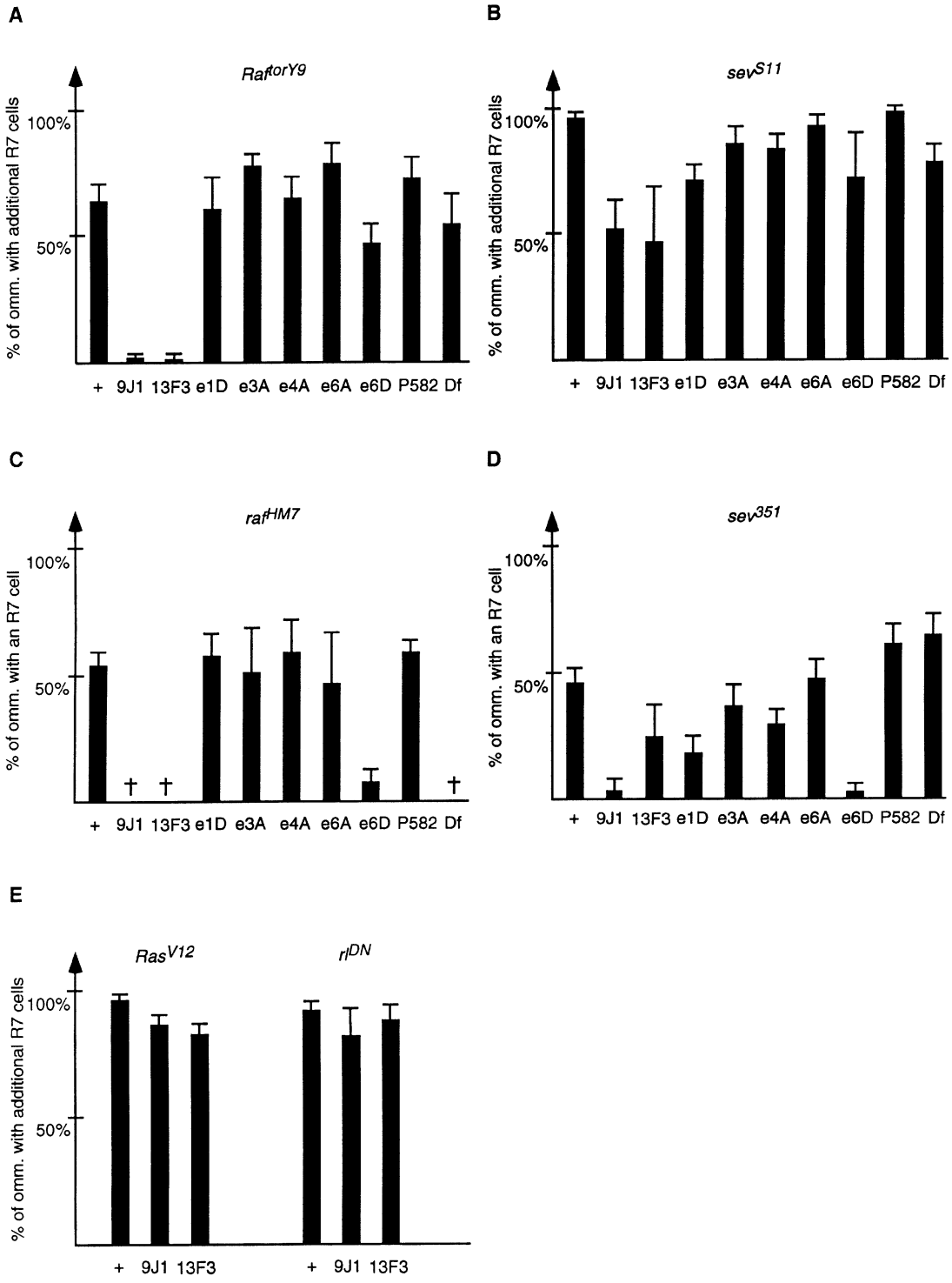

Fig. 4. Genetic interactions of *hsp83* alleles with gain-of-function and loss-of-function alleles of *raf* and *sev* and gain-of-function mutations in *Ras1* and *rl*. All seven EMS-induced *hsp83* alleles, the P element insertion line *P582* and a deficiency for *hsp83* [*Df(3L)M21*] were tested for an interaction with the gain-of-function alleles Raf^{torY9} (**A**) and sev^{S11} (**B**) or with the partial loss-of-function alleles raf^{HM7} (**C**) and sev^{351} (**D**). Eyes of flies heterozygous for either Raf^{torY9} (A) or sev^{S11} (B) and for one of the nine *hsp83* alleles were sectioned and the percentage of the ommatidia with more than one R7 cell was determined for each genotype. The multiple R7 phenotype of Raf^{torY9} is almost completely suppressed by the alleles *9J1* and *13F3*. *e1D*, *e4A* and *e6D* show a weak suppression and *e3A*, *e6A* and the P element *P582* do not suppress Raf^{torY9} detectably. The alleles show a similar, albeit generally weaker, interaction with sev^{S11}. The interaction between the *hsp83* alleles and the partial loss-of-function mutation raf^{HM7} (C) was quantified by determining the precentage of ommatidia with one R7 cell in raf^{HM7}/Y; *hsp83*/+ flies that had been reared at 18°C. At this temperature, hemizyous raf^{HM7} flies are semi-viable. Since the *9J1* and *13F3* alleles enhanced this semi-lethality to complete lethality, the eyes of these flies could not be analysed. Of all other alleles, only *e6D* weakly enhanced the semi-lethality (80% compared with the control) and the same is true for the enhancement of the eye phenotype. For the interaction with sev^{351}, the percentage of ommatidia with one R7 cell was determined in flies *w* sev^{d2} *P*[w^+ sev^{351}]/Y; *hsp83*/+. As in the case of the gain-of-function alleles of *raf* and *sev*, the relative degree of enhancement by each *hsp83* allele of the sev^{351} and raf^{HM7} phenotypes was similar. No significant suppression of the multiple R7 phenotype caused by the activation of Ras1 (Ras^{V12}, Fortini *et al.*, 1992) and Rl/MAP kinase (rl^{DN}, Brunner *et al.*, 1994) by the *9J1* and *13F3* alleles was observed (**E**). For each genotype, five eyes were analysed.

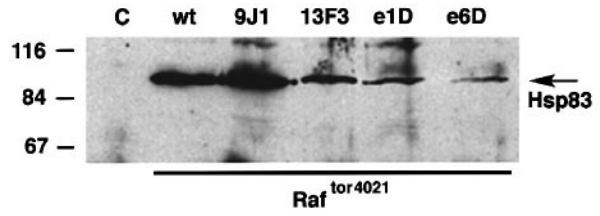

Fig. 5. Binding of Hsp83 to Raf. The *Raf^{tor4021}* construct was co-transfected into Schneider cells with constructs expressing epitope-tagged versions of wild-type Hsp83 or Hsp83 variants possessing the amino acid substitions found in either 9J1, 13F3, e1D or e6D. The first lane represents non-transfected control cells. Cell lysates were immunoprecipitated with anti-Raf antiserum and the immune complexes were subjected to Western blot analysis with the 9E10 antibody to detect the myc-tagged Hsp83 proteins.

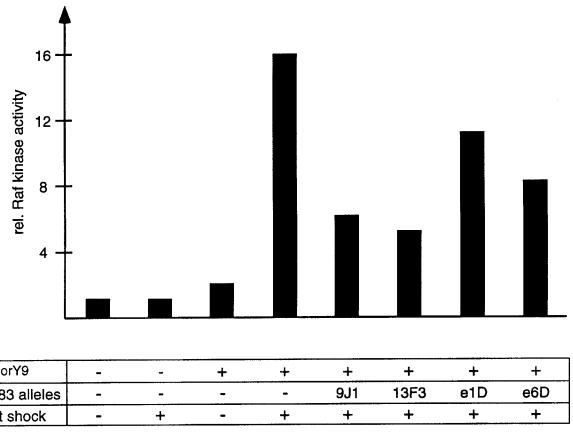

Fig. 6. Raf kinase activity is reduced by the mutant Hsp83 proteins. The Raf kinase activity in cell-free extracts from third instar larvae heterozygous for *sE-Raf^{torY9}* and one of the four *hsp83* alleles indicated as well as control larvae was determined by phosphorylation of the Raf-specific substrates GST–MEK1 and GST–Dsor1 in an *in vitro* kinase assay. To induce ubiquitious expression of the *sE-Raf^{torY9}* transgene, the larvae were heat-shocked prior to preparation of a cell-free larval extract. The extracts were incubated with recombinant kinase-inactive GST–MEK1 fusion protein and Raf activity was measured by the amount of ^{32}P incorporated into the substrate. The activity was quantified using a PhosphorImager (Molecular Dynamics) and is shown relative to the control larvae lacking the *sE-Raf^{torY9}* transgene. The assay was performed twice with recombinant kinase-inactive GST–MEK1 as well as once with recombinant kinase-inactive GST–Dsor1 as Raf-specific substrates and showed similar results.

exist as an 'aporeceptor complex', consisting of a single receptor molecule and an Hsp90 dimer, as well as several other molecules, such as hsp56/FKBP59 (Sanchez *et al.*, 1990). It is thought that the function of Hsp90 in this complex is to hold the receptor in a 'poised' state, in which it is able to bind the hormone ligand with high affinity, release the associated proteins and switch to its transcriptionally active state. Hsp90 dimers have also been shown to be tightly associated with the Raf serine/threonine kinase, in large multi-component complexes that include a different set of associated proteins to those found in the Hsp90–steroid receptor complexes (Stancato *et al.*, 1993; Wartmann and Davis, 1994). The function of Hsp90 in this complex, however, remains unclear.

We have isolated two antimorphic mutations in the *hsp83* gene, which encodes the *Drosophila* homologue of the Hsp90 protein. These mutations were isolated on the basis of their ability to dominantly suppress the formation of ectopic R7 cells in response to constitutive activation of the Raf kinase in the *Drosophila* eye. Both mutations also act as dominant enhancers of a hypomorphic *raf* allele, *raf^{HM7}*. This allele results in the reduced expression of a wild-type Raf kinase. Thus, we observe genetic interaction with both constitutively activated and wild-type forms of Raf kinase.

During *Drosophila* eye development, Raf acts in a signal transduction cascade that is initiated by activation of the Sev RTK. Another group previously has reported the isolation of five loss-of-function *hsp83* alleles on the basis of a genetic interaction with a temperature-sensitive *sev* allele (Simon *et al.*, 1991; Cutforth and Rubin, 1994). We found, however, that the majority of these *hsp83* alleles, as well as a deficiency for the locus, exhibit no genetic interaction with a different hypomorphic *sev* allele in which the kinase domain is intact. These results suggest that the genetic interaction observed between *sev* and *hsp83* is critically dependent on the temperature-sensitive mutation in the kinase domain, and may thus reflect the increased sensitivity of this conformationally unstable kinase to Hsp83 levels.

We do, however, observe weak genetic interactions between all three antimorphic *hsp83* alleles [two *Su(Raf)* and one *E(sev)*] and both gain- and loss-of-function *sev* alleles. This might be taken as evidence for a direct involvement of Hsp83 in Sev activation. However, these alleles show much stronger genetic interactions with *raf*, for which a physical association has also been shown, and result in a reduction in Raf kinase activity (Figure 6). Therefore, we propose that Hsp83 is involved directly only in Raf function, and that the weak genetic interactions between these *hsp83* alleles and *sev* is due to the requirement for Raf in Sev signalling.

An intriguing aspect of *hsp83* genetics is that, in general, the *Su(Raf)* and *E(Raf)* alleles complement each other, producing viable and, at least as far as eye development is concerned, completely wild-type flies. While this might be explained readily by mutations in two separate domains that disrupt the function of homodimers but not heterodimers, it is more difficult to envisage such a possibility in cases where both alleles appear to be antimorphic in nature. For example, the alleles *hsp83^{9J1}* and *hsp83^{e6D}* both act as 'dominant negatives' in their genetic interactions with *raf* and *sev,* but fully complement each other. Antimorphic mutations are often the result of one mutant molecule sequestering wild-type molecules in non-functional heterodimers. Clearly this cannot be the case in *hsp83^{9J1}/hsp83^{e6D}* animals, since both homo- and heterodimers would be non-functional and this allelic combination would be lethal.

We can offer two possible solutions to this paradox. Firstly, these alleles have only been shown to be antimorphic with respect to their interactions with Raf and Sev, but not with respect to viability, in which they act as normal recessive loss-of-function mutations (otherwise they would be dominantly lethal, obviously precluding their recovery). Perhaps heterodimers involving such an antimorphic allele do retain some function, sufficient for viability, but still detectable in our genetically sensitized assays. A second possibility is that even if heterodimers involving one wild-type and one antimorphic molecule are

non-functional, the heterodimers formed by two different antimorphic molecules are nevertheless functional, each mutation somehow making the molecule immune to the 'poisoning' effects of the other.

While the latter possibility may at first seem somewhat remote, we have obtained preliminary evidence that it may indeed partially account for the ability of two different antimorphic *hsp83* alleles to complement each other: whereas the eyes of $Raf^{torY9}/+$; $hsp83^{9J1}/+$ flies contain few if any ommatidia with additional R7 cells, a significant number of ommatidia in $Raf^{torY9}/+$; $hsp83^{9J1}/hsp83^{e6D}$ flies contain ectopic R7 cells (data not shown). This rather surprising observation suggests that 9J1:e6D heterodimers may indeed be much more effective in mediating Raf signalling than 9J1:+ heterodimers. It will be interesting to investigate the molecular basis of these results once the three-dimensional structure of Hsp90 and the sites of dimerization and interaction with other proteins have been determined.

In conclusion, we have presented here strong genetic evidence that Hsp83 plays an important role in signalling via the Raf kinase. How does Hsp83 facilitate Raf function? One possibility is that Hsp83 is needed merely for the maturation of the Raf protein. We do not consider it likely, however, that this is the only function of Hsp83 in Raf signalling, since it would be unlikely to account for the strong genetic and physical interactions we have observed between Raf and Hsp83. Another possibility is that Hsp83 may also help to assemble complexes consisting of both Raf and other signalling components, such as Ras and MEK. Both of these proteins have been observed to associate with the Raf–Hsp90 complex, and it has been suggested that the interaction with Ras is required for the Raf–Hsp90 complex to be translocated to the membrane (Leevers *et al.*, 1994; Stokoe *et al.*, 1994), while the interaction with MEK (Wartmann and Davis, 1994) is a prerequisite for this kinase to be a substrate of Raf. The constitutively activated Raf kinase we have used in these studies, however, is anchored to a transmembrane protein (Torso) and therefore reaches the plasma membrane independently of Ras (Dickson *et al.*, 1992). If the genetic interactions we observe between this activated Raf and Hsp83 are indeed due to a requirement for Hsp83 to assemble multi-component signalling complexes, then it is more likely that they disrupt the association with MEK rather than Ras.

While neither a chaperone nor template function for Hsp90 can be excluded on the basis of our data, the scenario we prefer is that Hsp90 facilitates Raf signalling in a manner similar to that proposed for its function in steroid receptor signalling, allowing it to switch rapidly to its active conformation once it reaches the plasma membrane. This model is consistent both with the strong requirement for Hsp90 in Raf signalling and also the observation that even a Raf protein targetted directly to the membrane still appears to require Hsp90 to achieve its active state.

Materials and methods

Genetics

The genetic screen for dominant modifiers of the Raf^{torY9} phenotype is described in detail in Dickson *et al.* (1996). In short, we screened ~200 000 F1 progeny of EMS-mutagenized males mated with females carrying the Raf^{torY9} fusion coding region under the transcriptional control of a single *sev* enhancer and the *hsp70* promoter, providing the two alleles $hsp83^{9J1}$ and $hsp83^{13F3}$. The P element insertion line *P582* was found in a collection of homozygous lethal P element insertions on the third chromosome (P.Deak and P.Maröy, unpublished data) by its failure to complement $hsp83^{9J1}$. The dominant suppression phenotype associated with the $hsp83^{9J1}$ and $hsp83^{13F3}$ chromosomes was mapped to chromosome 3, position 13 ± 4 cM using the markers *h*, *th*, *cu*, *sr* and *e*. The *hsp83* alleles were maintained as stocks balanced over either the *TM3*,Raf^{torY9} chromosome or a *TM6B* balancer. All the *hsp83* alleles are either embryonic or early larval lethal, with the excepetion of $hsp83^{13F3}$, which survives until the third larval instar stage. Flies carrying the raf^{HM7} mutation were raised at 18°C, all the other crossses were performed at 25°C. sev^{351} flies are sev^{d2} null mutants partially rescued by a P insertion carrying a *sev* cDNA construct in which the codons for Y1485 and W1486 have been replaced by alanine codons (B.Dickson and E.Hafen, unpublished data). The sev^{S11} mutant is described in Basler *et al.* (1991).

Scanning electron microscopy and histology

Adult flies for scanning microscopy were stored in 70% acetone before they were critical-point dried and coated for examination with a Hitachi S-4000 scanning electron microscope as described by Basler and Hafen (1988).

Molecular analysis

The DNA surrounding the *hsp83* locus was isolated using the plasmid rescue technique (Mlodzik *et al.*, 1990). cDNAs were isolated from a λgt10 third instar eye–antennal disc library prepared by A.Cowman. The largest cDNA clone was subcloned into M13 and the ends were sequenced. A database search with the partial cDNA sequence indicated that it was identical to the sequence of the *hsp83* gene (Hackett and Lis, 1983). To determine the DNA alterations present in the $hsp83^{9J1}$ and $hsp83^{13F3}$ alleles, genomic DNA was amplified in several independent PCRs from $hsp83^{9J1}/TM3,Raf^{torY9}$, $hsp83^{13F}/TM3,Raf^{torY9}$ flies and as a control from an independent suppressor, $Su(Raf)3B/TM3,Raf^{torY9}$, representing the parental $hsp83^{+}$ chromosome. Fragments were amplified using leader- and trailer-specific primers, subcloned into M13 and sequenced in one direction. We also amplified DNA from $hsp83^{9J1}/hsp83^{e3A}$ and $hsp83^{13F3}/hsp83^{e3A}$ flies and used a polymorphism present in the $hsp83^{e3A}$ allele (Cutforth and Rubin, 1994) to distinguish this from $hsp83^{9J1}$ and $hsp83^{13F3}$ products.

Germline transformation

The construct used for rescue experiments was a 2.7 kb cDNA fragment cloned into a modified pW8 transformation vector (Klemenz *et al.*, 1987) containing the inducible *hsp70* promoter and two copies of a 1.2 kb *sev* enhancer element (Basler *et al.*, 1989). The pW82 construct (Wohlwill and Bonner, 1991) contained a 7.5 kb *Bam*HI–*Bgl*II fragment including the *hsp83* gene as well as the adjancent gene (*T2*) cloned into the transformation vector CaSpeR (Pirotta, 1988). Transgenic flies were generated as described by Basler *et al.* (1991).

Schneider cell transfections

The Hsp83-myc fusion construct was generated by introducing a *Bam*HI site at the 3′ end of the *hsp83* open reading frame via PCR mutagenesis and by then inserting an oligonucleotide encoding the c-myc epitope (Evan *et al.*, 1985) between this *Bam*HI site and an *Sph*I site in the polylinker of the vector. These manipulations added codons for the amino acid sequence GSQGTEQKLISEEDLN to the end of the *hsp83* ORF. In this fusion construct, four *hsp83* mutations (*9J1*, *13F3*, *e1D* and *e6D*) were introduced via *in vitro* mutagenesis using the following oligonucleotides: CAGATCCTTGGAGTCGA (*9J1*), CTCATAGCAGATCTTGT (*13F3*), AAGCGTTCAAGATCAAC (*e1D*) and CTGACCCTTCACGGAGA (*e6D*). These cDNAs were then subcloned into the expression vector pPAC *Bam*HI containing the actin5C promoter (Krasnow *et al.*, 1989). The $Raf^{tor4021}$ construct includes the *sev* enhancer and the heat-inducible *hsp70* promoter, which permits heat induction. Schneider S2 cells were co-transfected by the calcium chloride method (Ashburner, 1989), incubated for 72 h and then prepared for further examinations. The Schneider S2 cells were kept in Schneider cell medium (Gibco) supplemented with 10% fetal calf serum.

Immunoprecipitations and Western blotting

For immunoprecipitations, the cells were heat shocked for 1 h at 37°C, allowed to recover for 3–4 h, washed once with phosphate-buffered

saline (PBS) and lysed in buffer A [20 mM Tris, pH 7.4, 137 mM NaCl, 2 mM EDTA, 2 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin, 1% Triton X-100, 10% glycerol and 25 mM β-glycerophosphate] (Wartmann and Davis, 1994). Lysates were clarified by centrifugation and incubated for 2 h at 4°C with 4 μl of anti-Raf antiserum (F.Sprenger and C.Nüsslein-Volhard, unpublished) and protein A–Sepharose (Sigma). Immunoprecipitates were washed once with buffer A, twice with buffer D (buffer A supplemented with 0.1% SDS and 0.5% sodium deoxycholate), once with buffer E (10 mM Tris, pH 7.4 and 25 mM β-glycerophosphate) and then boiled for 5 min in SDS sample buffer. The samples were then fractionated by electrophoresis on an 8% SDS–PAGE and analysed by Western blotting. The blots were probed with a 1:5000–1:1000 dilution of anti-myc antibody (BAbCO). The blots were developed using the ECL kit (Amersham).

Cell-free in vitro kinase assay

For each assay, six larvae (with or without heat shock) were washed three times with ice-cold PBS and twice with kinase buffer (50 mM Tris–HCl, pH 7.4, 50 mM NaCl, 10 mM $MnCl_2$, 2 mM dithiothreitol, 25 mM β-glycerophosphate, 25 mM NaF, 1 mM PMSF, 1 mM leupeptin, 1 mM pepstatin, 1 mM benzamidin, 200 KIE/ml trasylol) prior to lysis. Larvae were homogenized in 250 μl of kinase buffer by six strokes using a homogenizer adapted with a plastic Eppendorf tube pestle. The total larval extract was adjusted to 0.5% NP-40 and incubated for 20 min at 4°C by end-over-end rotation. The obtained lysate was cleared by high-speed centrifugation twice for 10 min at 4°C. For measurement of Raf kinase activity, 200 μl of cell-free larval extract was agitated gently for 20 min at 30°C with 5 μg of recombinant kinase-inactive GST–MEK1 or 2 μg of recombinant kinase-inactive GST–Dsor1 in the presence of 15 μCi of [γ-^{32}P]ATP (Amersham) and 5 μM ATP per reaction.

To isolate the *in vitro* phosphorylated recombinant GST–MEK1 or GST–Dsor1 substrate, the kinase reaction was incubated subsequently for 1 h with 10 μl of washed glutathione beads. The beads were washed three times with kinase buffer containing 0.5% NP-40 and resuspended in 50 μl of SDS sample buffer. The samples were heated at 95°C for 5 min. After SDS–PAGE (10%), the quantification of radioactivity incorporated into GST–MEK or GST–Dsor1 substrates was carried out with a PhosphorImager (Molecular Dynamics).

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References

Aitken,A. (1995) 14-3-3 proteins on the MAP. *Trends Biochem. Sci.*, **20**, 95–97.

Ashburner,M. (1989) *Drosophila: A Laboratory Handbook*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Basler,K. and Hafen,E. (1988) Control of photoreceptor cell fate by the sevenless protein requires a functional tyrosine kinase domain. *Cell*, **54**, 299–312.

Basler,K., Siegrist,P. and Hafen,E. (1989) The spatial and temporal expression pattern of *sevenless* is exclusively controlled by gene-internal elements. *EMBO J.*, **8**, 2381–2386.

Basler,K., Christen,B. and Hafen,E. (1991) Ligand-independent activation of the sevenless receptor tyrosine kinase changes the fate of cells in the developing *Drosophila* eye. *Cell*, **64**, 1069–1082.

Biggs,W.H.,III, Zavitz,K.H., Dickson,B., van der Straten,A., Brunner,D., Hafen,E. and Zipursky,S.L. (1994) The *Drosophila rolled* locus encodes a MAP kinase required in the sevenless signal transduction pathway. *EMBO J.*, **13**, 1628–1635.

Blackmann,R.K. and Meselson,M. (1986) Interspecific nucleotide sequence comparisons used to identify regulatory and structural features of the *Drosphila hsp82* gene. *J. Mol. Biol.*, **188**, 499–515.

Bohen,S.P. and Yamamoto,K.R. (1993) Isolation of Hsp90 mutants by screening for decreased steroid receptor function. *Proc. Natl Acad. Sci. USA*, **90**, 11424–11428.

Bohen,S.P. and Yamamoto,K.R. (1994) Modulation of steroid receptor signal transduction by heat shock proteins. In Morimoto,R.I., Tissères,A. and Georgopolous,C. (eds), *The Biology of Heat Shock Proteins and Molecular Chaperones*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 313–334.

Brunner,D., Oellers,N., Szabad,J., Biggs,W.H.,III, Zipursky,S.L. and Hafen,E. (1994) A gain of function mutation in *Drosophila* MAP kinase activates multiple receptor tyrosine kinase signalling pathways. *Cell*, **76**, 875–888.

Catelli,M.G., Binart,N., Jung,T.I., Renoir,J.M., Baulieu,E.E., Feramisco,J.R. and Welch,W.J. (1985) The common 90-kd protein component of non-transformed '8S' steroid receptors is a heat-shock protein. *EMBO J.*, **4**, 3131–3135.

Chang,H.C., Solomon,N.M., Wassarman,D.A., Karim,F.D., Therrien,M., Rubin,G.M. and Wolff,T. (1995) *phyllopod* functions in the fate determination of a subset of photoreceptors in *Drosophila*. *Cell*, **80**, 463–472.

Cutforth,T. and Rubin,G.M. (1994) Mutations in Hsp83 and cdc37 impair signaling by the sevenless receptor tyrosine kinase in *Drosophila*. *Cell*, **77**, 1027–1036.

Dickson,B. (1995) Nuclear factors in sevenless signalling. *Trends Genet.*, **11**, 106–111.

Dickson,B., Sprenger,F., Morrison,D. and Hafen,E. (1992) Raf functions downstream of Ras1 in the Sevenless signal transduction pathway. *Nature*, **360**, 600–603.

Dickson,B.J., Domínguez,M., van der Straten,A. and Hafen,E. (1995) Control of *Drosophila* photoreceptor cell fates by phyllopod, a novel nuclear protein acting downstream of the Raf kinase. *Cell*, **80**, 453–462.

Dickson,B.J., van der Straten,A., Domínguez,M. and Hafen,E. (1996) Mutations modulating Raf signaling in *Drosophila* eye development. *Genetics*, **142**, 163–171.

Domínguez,M. and Hafen,E. (1996) Genetic dissection of cell fate specification in the developing eye of *Drosophila*. *Semin. Cell Dev. Biol.*, **7**, 219–226.

Evan,G.I., Lewis,G.K., Ramsey,G. and Bishop,J.M. (1985) Isolation of monoclonal antibodies specific for human c-*myc* proto-oncogene product. *Mol. Cell. Biol.*, **5**, 3610–3616.

Fortini,M.E., Simon,M.A. and Rubin,G.M. (1992) Signalling by the *sevenless* protein tyrosine kinase is mimicked by Ras1 activation. *Nature*, **355**, 559–561.

Greenwald,I. and Rubin,G.M. (1992) Making a difference: the role of cell–cell interactions in establishing separate identities for equivalent cells. *Cell*, **68**, 271–281.

Hackett,R.W. and Lis,J.T. (1983) Localization of the hsp83 transcript within a 3292 nucleotide sequence from the 63B heat shock locus of *D.melanogaster*. *Nucleic Acids Res.*, **11**, 7011–7030.

Herbst,R., Carroll,P.M., Allard,J.D., Schilling,J., Raabe,T. and Simon,M.A. (1996) Daughter of sevenless is a substrate of the phosphotyrosine phosphatase Corkscrew and functions during Sevenless signaling. *Cell*, **85**, 899–909.

Holgado-Madruga,M., Emlet,D.R., Moscatello,D.K., Godwin,A.K. and Wong,A.J. (1996) A Grb2-associated docking protein in EGF- and insulin-receptor signalling. *Nature*, **379**, 560–564.

Klemenz,R., Weber,U. and Gehring,W.J. (1987) The *white* gene as a marker in a new P-element vector for gene transfer in *Drosophila*. *Nucleic Acids Res.*, **15**, 3947–3959.

Krasnow,M.A., Saffman,E.E., Kornfeld,K. and Hogness,D.S. (1989) Transcriptional activation and repression by Ultrabithorax proteins in cultured *Drosophila* cells. *Cell*, **57**, 1031–1043.

Leevers,S.J., Paterson,H.F. and Marshall,C.J. (1994) Requirement for Ras in Raf activation is overcome by targeting Raf to the plasma membrane. *Nature*, **369**, 411–414.

Lu,X., Melnick,M.B., Hsu,J.C. and Perrimon,N. (1994) Genetic and molecular analysis of mutations involved in *Drosophila* raf signal transduction. *EMBO J.*, **13**, 2592–2599.

Marais,R., Light,Y., Paterson,H.F. and Marshall,C.J. (1995) Ras recruits Raf-1 to the plasma membrane for activation by tyrosine phosphorylation. *EMBO J.*, **14**, 3136–3145.

Marshall,C.J. (1994) MAP kinase kinase kinase, MAP kinase kinase and MAP kinase. *Curr. Opin. Genet. Dev.*, **4**, 82–89.

Minami,Y., Kimura,Y., Kawasaki,H., Suzuki,K. and Yahara,I. (1994) The carboxy-terminal region of mammalian HSP90 is required for its dimerization and function *in vivo*. *Mol. Cell. Biol.*, **14**, 1459–1464.

Miyata,Y. and Yahara,I. (1992) The 90-kDa heat shock protein, HSP90, binds and protects casein kinase II from self-aggregation and enhances its kinase activity. *J. Biol. Chem.*, **267**, 7042–7047.

Mlodzik,M., Hiromi,Y., Weber,U., Goodman,C.S. and Rubin,G.M. (1990) The *Drosophila seven-up* gene, a member of the steroid receptor gene superfamily, controls photoreceptor cell fates. *Cell*, **60**, 211–224.

O'Connor,D. and Lis,J.T. (1981) Two closely linked transcription units within the 63B heat shock puff locus of *D.melanogaster* display strikingly different regulation. *Nucleic Acids Res.*, **9**, 5075–5092.

Olivier,J.P., Raabe,T., Henkemeyer,M., Dickson,B., Mbamalu,G., Margolis,B., Schlessinger,J., Hafen,E. and Pawson,T. (1993) A *Drosophila* SH2–SH3 adaptor protein implicated in coupling the Sevenless tyrosine kinase to an activator of Ras guanine nucleotide exchange, Sos. *Cell*, **73**, 179–191.

Parsell,D.A. and Lindquist,S. (1993) The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. *Annu. Rev. Genet.*, **27**, 437–496.

Pawson,T. (1995) Protein modules and signalling networks. *Nature*, **373**, 573–580.

Pirotta,V. (1988) Vectors for P-mediated transformation in Drosophila. In Rodriguez,R.L. and Denhardt,D.T. (eds), *Vectors. A Survey of Molecular Cloning Vectors and Their Uses*. Butterworths, pp. 438–456.

Raabe,T., Riesgo-Escovar,J., Liu,X., Bausenwein,B.S., Deak,P., Maröy,P. and Hafen,E. (1996) DOS, a novel Pleckstrin homology domain-containing protein required for signal transduction between Sevenless and Ras1 in *Drosophila*. *Cell*, **85**, 911–920.

Rogge,R.D., Karlovich,C.A. and Banerjee,U. (1991) Genetic dissection of a neurodevelopmental pathway: *son of sevenless* functions downstream of the *sevenless* and EGF receptor tyrosine kinases. *Cell*, **64**, 39–48.

Sanchez,E.R., Faber,L.E., Henzel,W.J. and Pratt,W.B. (1990) The 56–59-kilodalton protein identified in untransformed steroid receptor complexes is a unique protein that exists in cytosol in a complex with both the 70- and 90-kilodalton heat shock proteins. *Biochemistry*, **29**, 5145–5152.

Simon,M.A., Bowtell,D., Dodson,G.S., Laverty,T.R. and Rubin,G.M. (1991) Ras1 and a putative guanine nucleotide exchange factor perform crucial steps in signaling by the sevenless protein tyrosine kinase. *Cell*, **67**, 701–716.

Simon,M.A., Dodson,G.S. and Rubin,G.M. (1993) An SH3–SH2–SH3 protein is required for p21Ras1 activation and binds to sevenless and Sos proteins *in vitro*. *Cell*, **73**, 169–177.

Stancato,L.F., Chow,Y.H., Hutchison,K.A., Perdew,G.H., Jove,R. and Pratt,W.B. (1993) Raf exists in a native heterocomplex with hsp90 and p50 that can be reconstituted in a cell-free system. *J. Biol. Chem.*, **268**, 21711–21716.

Stokoe,D., Macdonald,S.G., Cadwallader,K., Symons,M. and Hancock,J.F. (1994) Activation of Raf as a result of recruitment to the plasma membrane. *Science*, **264**, 1463–1467.

Therrien,M., Chang,H.C., Solomon,N.M., Karim,F.D., Wassarman,D.A. and Rubin,G.M. (1995) KSR, a novel protein kinase required for RAS signal transduction. *Cell*, **83**, 879–888.

Tsuda,L. *et al*. (1993) A protein kinase similar to MAP kinase activator acts downstream of the raf kinase in *Drosophila*. *Cell*, **72**, 407–414.

Van Vactor,D.L.J., Cagan,R.L., Krämer,H. and Zipursky,S.L. (1991) Induction in the developing compound eye of *Drosophila*: multiple mechanisms restrict R7 induction to a single retinal precursor cell. *Cell*, **67**, 1145–1155.

Wartmann,M. and Davis,R.J. (1994) The native structure of the activated Raf protein kinase is a membrane-bound multi-subunit complex. *J. Biol. Chem.*, **269**, 6695–6701.

Wohlwill,A.D. and Bonner,J.J. (1991) Genetic analysis of chromosome region 63 of *Drosophila melanogaster*. *Genetics*, **128**, 763–775.

Xu,Y. and Lindquist,S. (1993) Heat-shock protein hsp90 governs the activity of pp60v-src kinase. *Proc. Natl. Acad. Sci. USA*, **90**, 7074–7078.

Zipursky,S.L. and Rubin,G.M. (1994) Determination of neuronal cell fate: lessons from the R7 neuron of *Drosophila*. *Annu. Rev. Neurosci.*, **17**, 373–397.

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