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

van der Straten, A; Rommel, C; Dickson, B; Hafen, E
The heat shock protein 83 (Hsp83) is required for Raf-mediated signalling in Drosophila

Abstract

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

Alexandra van der Straten, Christian Rommel\(^1\), Barry Dickson and Ernst Hafen

Zoologisches Institut, Universit"at Z"urich, Winterthurerstrasse 190, CH-8057 Z"urich and \(^1\)Institut f"ur Medizinische Virologie, Gloristrasse 30, CH-8028 Z"urich, Switzerland

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; Dominguez 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., 1991).
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 constitutively 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; Cutford 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 Raf9Y9 flies (described in Dickson et al., 1992), but differ markedly in their interactions with a hypomorphic *raf* allele, *raf*<sup>64T</sup>. Hemizygous *raf*<sup>64T</sup> 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*<sup>64T</sup> phenotype, but whereas the *raf*<sup>64T</sup> allele becomes completely lethal in a *rl*<sup>+/+</sup> background, no reduction of viability is seen at all in a *phyl*<sup>+/+</sup> background. The interaction with *raf*<sup>64T</sup> 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<sup>torY9</sup> and *raf*<sup>64T</sup>, 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<sup>torY9</sup> phenotype we isolated, completely eliminating all additional R7 cells (Figure 1C and G; Figure 4A). The strong suppression is specific for Raf<sup>torY9</sup>, since 9J1 and 13F3 do not strongly suppress the rough eye phenotype caused by the constitutive activation of Ras1 (see-Ras<sup>1/2</sup>) or RI/MAP kinase (p<sup>DN</sup>) (Figure 4E). Furthermore, as with rl, the *raf*<sup>64T</sup> mutation becomes lethal in a Su(Raf)3A<sup>+/+</sup> 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<sup>torY9</sup>, we also identified a lethal P element insertion, *P<sup>582</sup>* (P.Deak and P.Maróy, 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 *P<sup>582</sup>* insertion (Figure 2A) and determined that this P element lies within the 5'-untranslated region (5'-UTR) of the hsp83 gene (Hackett and Liu, 1983). A construct containing 7.5 kb of genomic DNA from this region, including both hsp83 and an adjacent transcript (Wohlfwill and Bonner, 1991), rescued the lethality of Su(Raf)3A mutations in transgenic flies. Furthermore, introducing this construct into a Raf<sup>torY9</sup>; *Su(Raf)3A<sup>+/+</sup>* 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 Liu, 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<sup>torY9</sup>; *Su(Raf)3A<sup>+/+</sup>* background (Figure 1D and H).

Finally, we cloned and sequenced the hsp83 gene from both Su(Raf)3A<sup>9J1</sup> and Su(Raf)3A<sup>13F3</sup> 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 *P<sup>582</sup>* insertion nor deficiencies that completely remove enhance the hsp83 gene to the cytological interval 63A1; C1. In addition to this construct into a Raf<sup>torY9</sup>; *Su(Raf)3A<sup>+/+</sup>* 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 Liu, 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<sup>torY9</sup>; *Su(Raf)3A<sup>+/+</sup>* background (Figure 1D and H).

Finally, we cloned and sequenced the hsp83 gene from both Su(Raf)3A<sup>9J1</sup> and Su(Raf)3A<sup>13F3</sup> 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 *P<sup>582</sup>* insertion nor deficiencies that completely remove the hsp83 gene act as dominant suppressors of the Raf<sup>torY9</sup> phenotype, we infer that both EMS-induced mutations are antimorphic in nature.
Hsp83 is required for Raf signalling

Fig. 1. The Raf

\textsuperscript{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\textsuperscript{torY9}/+(B and F), Raf\textsuperscript{torY9}/; Su(Raf)3A\textsuperscript{9J1}/+(C and G), sE-hsp83+/; Su(Raf)3A\textsuperscript{9J1}, Raf\textsuperscript{torY9}/+(D and H). The Raf\textsuperscript{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\textsuperscript{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).

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 withraf. 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\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{ts} screen, in which only 30 000 flies were screened. Conversely, hypomorphic E(sev) alleles, recovered at high frequency (1/6000) in the sev\textsuperscript{ts} screen, do not interact with Raf\textsuperscript{torY9} and thus could not have been recovered in the Raf\textsuperscript{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

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\textsuperscript{e4A} and hsp83\textsuperscript{e6D}, do not complement the Su(Raf) allele hsp83\textsuperscript{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.
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\textsuperscript{torY9} kinase activity. Larvae heterozygous for Raf\textsuperscript{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\textsuperscript{torY9} transgene. Cell-free extracts were prepared and incubated with recombiantin kinase-inactive GST–MEK. Upon heat induction, the Raf kinase activity in Raf\textsuperscript{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\textsuperscript{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 Bohm and Yamamoto, 1994). In the absence of a ligand, many steroid receptors have been shown to bind Raf, although compared with wild-type Hsp83 the activated form of Raf (Raftor\textsuperscript{4021}) is identical to Raf\textsuperscript{torY9} except that it contains a different amino acid substitution 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 (Evans 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\textsuperscript{torY9} 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\textsuperscript{9J1} and hsp83\textsuperscript{13F3}, as well as two of the E(sev) alleles, hsp83\textsuperscript{e1D} and hsp83\textsuperscript{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.
Hsp83 is required for Raf signalling

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 percentage of ommatidia with one R7 cell in raf HM7/Y; hsp83/1 flies that had been reared at 18°C. At this temperature, hemizygous raf HM7 flies are semi-viable. Since the 9J1 and 13F3 alleles enhanced this semi-viability to complete lethality, the eyes of these flies could not be analysed. Of all other alleles, only e6D weakly enhanced the semi-viability (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 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 RI/MAP kinase (rlDN, Brunner et al., 1994) by the 9J1 and 13F3 alleles was observed (E). For each genotype, five eyes were analysed.
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, raf9MC. 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 hsp839J1 and hsp83e6D 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 hsp839J1/hsp83e6D 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(IT281)+; hsp82(T281)+ flies contain few if any ommatidia with additional R7 cells, a significant number of ommatidia in Raf(IT281)+; hsp83(T281)/hsp83(T280) flies contain ectopic R7 cells (data not shown). This rather surprising observation suggests that 91J:6D heterodimers may indeed be much more effective in mediating Raf signalling than 91J:1 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 (Leever et al., 1994; Stokoe et al., 1994), while the interaction with MEK (Wartman 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 (Toso) 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 Hsp83 facilitates Raf signalling in a manner similar to that proposed for its function in steroid receptor signalling, allowing it to switch rapidly and in a manner similar to that proposed for its function in the scenario we prefer is that Hsp90 facilitates Raf signalling polylinker of the vector. These manipulations added codons for the 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(IT281) 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(IT281) fusion coding region under the transcriptional control of a single sev enhancer and the hsp67B promoter, providing the two alleles hsp83(9J1) and hsp83(9J3). 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.Marth, unpublished data) by its failure to complement hsp82(T281). 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,RFa(3) chromosome or a TM6B balancer. All the hsp83 alleles are the same embryonic or early larval lethal, with the exception of hsp83(13F3), which survives until the third larval instar stage. Flies carrying theraf(Y9) mutation were raised at 18°C, all the other crosses were performed at 25°C. sev(Y9) flies are sev2 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 (V.Dickson and E.Hafen, unpublished data). The sev(Y9) mutant is described in Basler et al. (1991).

Scanning electron microscopy and histology

Adult flies for scanning microscopy were stored in 70% acetone before 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 (Leever et al., 1994; Stokoe et al., 1994), while the interaction with MEK (Wartman 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 (Toso) 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 Hsp83 facilitates Raf signalling in a manner similar to that proposed for its function in steroid receptor signalling, allowing it to switch rapidly and in 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(IT281) 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(IT281) fusion coding region under the transcriptional control of a single sev enhancer and the hsp67B promoter, providing the two alleles hsp83(9J1) and hsp83(9J3). 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.Marth, unpublished data) by its failure to complement hsp82(T281). 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,RFa(3) chromosome or a TM6B balancer. All the hsp83 alleles are the same embryonic or early larval lethal, with the exception of hsp83(13F3), which survives until the third larval instar stage. Flies carrying theraf(Y9) mutation were raised at 18°C, all the other crosses were performed at 25°C. sev(Y9) flies are sev2 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 (V.Dickson and E.Hafen, unpublished data). The sev(Y9) mutant is described in Basler et al. (1991).

Scanning electron microscopy and histology

Adult flies for scanning microscopy were stored in 70% acetone before dimerization and interaction with other proteins have been determined.
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 antisum (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 MnCl₂, 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 recombinant kinase-extractive GST–MEK1 or 2 µg of recombinant kinase-inactive GST–Dsor1 in the presence of 15 µCi of [γ-32P]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–MEK1 or GST–Dsor1 substrates was carried out with a PhosphorImager (Molecular Dynamics).

**Acknowledgements**

We thank N.Oellers for help with the Hsp83 binding studies and for providing the GST–Dso1 protein. We are grateful to K.K.Mordecai for her support, C.Hugentobler and P.Faller for technical assistance, F.Sprenger and C.Nüsslein-Volhard for the anti-Raf antisum, G.Rubin and T.Courtice for the El(sey) alleles and S.Leewers as well as members of the Hafen lab for comments on the manuscript. E.H. is supported by a grants from the Swiss National Science Foundation and from the Human Frontiers of Science Organization.

**References**


