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Dual function of Ras in Raf activation

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SUMMARY

The small guanine nucleotide binding protein p21^{Ras} plays an important role in the activation of the Raf kinase. However, the precise mechanism by which Raf is activated remains unclear. It has been proposed that the sole function of p21^{Ras} in Raf activation is to recruit Raf to the plasma membrane. We have used *Drosophila* embryos to examine the mechanism of Raf (Draf) activation in the complete absence of p21^{Ras} (Ras1). We demonstrate that the role of Ras1 in Draf activation is not limited to the translocation of Draf to the membrane through a Ras1-Draf association. In addition, Ras1 is essential for the activation of an additional factor which in turn activates Draf.

Key words: Ras, Raf, Receptor tyrosine kinase, *Drosophila melanogaster*

INTRODUCTION

Signaling through the evolutionarily conserved cassette of molecules including p21^{Ras} (Ras), Raf and the mitogen-activated protein kinase (MAPK) plays an essential role in mediating proliferation and development in many organisms (reviewed by Avruch et al., 1994; Marshall, 1994; Moodie and Wolfman, 1994). Although Raf has been shown to be essential for transducing signals downstream of activated Ras, it is not known precisely how Raf is activated (reviewed by Morrison and Cutler, 1997). The current model states that the sole function of Ras in Raf activation is to translocate Raf to the plasma membrane, where Raf activation is regulated by an unknown factor(s) (Leevers et al., 1994; Stokoe et al., 1994). Two basic observations constitute the basis of this model. First, purified Raf-1 cannot be activated by Ras-GTP in vitro (Moodie et al., 1993; Vojtek et al., 1993). Second, in cultured cells, Raf-1 can be activated by being targeted to the membrane without co-transfection of activated Ras. This activation of membrane-targeted Raf cannot be inhibited by a dominant negative form of Ras (Leevers et al., 1994; Stokoe et al., 1994).

All Raf proteins share three Conserved Regions, CR1, CR2, and CR3 (Daum et al., 1994). CR1 and CR2 are located in the N-terminal regulatory region, which acts to suppress the catalytic activity of the C-terminal CR3 kinase domain. Removal of the N terminus (including CR1 and CR2) results in constitutive activation of Raf-1 in mammalian cells (Heidecker et al., 1990; Stanton et al., 1989). The activation of Raf requires its direct interaction with Ras in its activated GTP-bound form, and two regions within CR1 have been identified that bind Ras: the Ras-binding domain (RBD; Gorman et al., 1996; Nassar et al., 1995; Scheffler et al., 1994; Vojtek et al., 1993) and a cysteine-rich domain (CRD; Hu et al., 1995; Mott et al., 1996). The RBD associates with the Ras effector domain with high affinity, and Arg89 located in the RBD of Raf-1 is a key residue that mediates this interaction (Block et al., 1996). A mutation in this residue, R89L, abolishes the association between Ras and Raf-1, as well as Ras-dependent activation of Raf-1 (Fabian et al., 1994). The CRD also associates with Ras through residues different from those that contact the RBD (Hu et al., 1995). Interestingly, it was demonstrated that the CRD:Ras interaction only occurs once the association of RBD with Ras has occurred (Drugan et al., 1996).

The *Drosophila* Draf protein is structurally and functionally homologous to mammalian Raf-1. Human RAF-1 is 46% identical in amino acid sequence to Draf, and is able to substitute in *Drosophila* for Draf for viability and signal transduction (Ambrosio et al., 1989; Casanova et al., 1994; A. Brand, X. Lu, and N. Perrimon, unpublished data). A *Drosophila* mutation, *Draf*^{C110}, associated with a reduced level of Draf activity, has been isolated that cannot support the survival of the animal (Melnick et al., 1993). This mutation is an amino acid change in Arg217, equivalent to Arg89 of Raf-1. The *Draf*^{C110} R217L mutation prevents the Draf:Ras1 interaction, suggesting that like Arg89 in Raf-1, Arg217 is essential for Ras1:Draf interaction (Hou et al., 1995). Dominant intragenic suppressors of *Draf*^{C110} have been identified that restore viability to *Draf*^{C110} flies (Lu et al., 1994). Two of these suppressors, *Su2* and *Su3*, identify mutations P308L and F290I in the CRD of Draf, respectively. When the analogous *Draf*^{C110} mutation along with either of its intragenic suppressors were introduced into mammalian Raf-1, it was shown that the suppressors do not function to restore the lost Ras:Raf association. Rather, they increase the basal level activity of Raf-1 (Cutler and Morrison, 1997).

To further understand the mechanism of Raf activation by Ras, we decided to examine the activity of a number of Raf mutations in the complete absence of Ras activity. These experiments are feasible in *Drosophila* because it is possible to generate eggs completely devoid of Ras1 or Draf activity using the mosaic 'FLP-DFS' technique (Chou and Perrimon, 1996). This is achieved following generation of germline mosaics that allow production of eggs derived from germline cells homozygous for either Ras1 or Draf protein null mutations. The embryos derived from females that lack maternal *Draf* or *Ras1* activities are referred to as *Draf* or *Ras1* embryos, respectively. In the absence of *Ras1* or *Draf* gene activity, receptor tyrosine kinase (RTK) signaling pathways in the embryo are not able to signal. In particular, the Torso (Tor) RTK signaling pathway, which is responsible for determination of embryonic terminal cell fates (reviewed by Duffy and Perrimon, 1994), is the first RTK pathway to be affected. In the absence of Tor RTK activity, downstream target genes such as *tailless* (*tll*) and *huckebein* (*hkb*) are not activated. *tll* (Pignoni et al., 1990, 1992) is an excellent molecular 'read-out' of Tor signaling because both the domain and level of *tll* expression in the posterior region of the embryo reflect the strength of Tor activation. At the posterior terminus in wild-type embryos, *tll* is expressed between 0 to 15% egg length (EL). If Tor signaling is decreased the domain of *tll* expression is reduced (<15% EL), and an increase in Tor signaling activity expands the *tll* expression domain towards the middle of the embryo (>15% EL). Draf plays a central role in Tor signal transduction because embryos lacking maternal *Draf* gene activity have phenotypes identical to those of *tor* null embryos, resulting in the complete absence of posterior *tll* expression. Weaker *Draf* alleles exhibit reduced levels of *tll* expression (Melnick et al., 1993). Therefore, visualization of *tll* expression allows one to determine the level of activity of Tor, Draf, or its dowsntream signal transducer (see Hou et al., 1995; Li et al., 1997).

Previously, it has been proposed that N-terminal truncation or membrane targeting of Raf results in Ras-independent 'constitutive activation' of Raf (Leevers et al., 1994; Stokoe et al., 1994). We have reexamined the signaling activity of an N-terminally truncated Draf (DrafΔN) mutation expressed in embryos completely devoid of *Ras1* activity. We demonstrate, contrary to the current model, that Ras1 is necessary for the activation of DrafΔN. Further, we show that the activity of membrane-targeted DrafΔN is still sensitive to the presence of Ras1. Finally, we examined the role of Ras1 in Draf activation using point mutations in Draf that disrupt its association with Ras1. We find that $Draf^{C110}$ in combination with one of its intragenic suppressors, *Su3*, is still regulated by Ras1 and Tor, although *Su3* does not restore the $Draf^{C110}$:Ras1 molecular interaction. The unregulated enzymatic activity of Raf-1, due to the *Su3* mutation (Cutler and Morrison, 1997), is not sufficient for the mutant Draf to transduce Tor signals, suggesting that Ras1, which is not physically interacting with the mutant Draf protein, is required for the activation of the mutant Draf. Taken together, these results suggest that Ras has an essential function in Raf activation beyond translocating Raf to the plasma membrane.

MATERIALS AND METHODS

Fly stocks and production of germline clone embryos

tor null mutant embryos were collected from females homozygous for tor^{XR1}, a protein null allele of *tor* (Sprenger et al., 1993). The 'FLP-DFS' technique (Chou and Perrimon, 1992, 1996) was used to produce embryos derived from germline clones homozygous for $D\text{-}Raf^{11\text{-}29}$, a protein null allele of *D-Raf* (Sprenger et al., 1993), and $Ras1^{\Delta C40B}$, a protein null allele of *Ras1* (Hou et al., 1995).

Embryos derived from germ cells doubly homozygous for $Draf^{(R217L,\ F290I)}$ and *tor* were derived from females homozygous for $Draf^{(R217L,\ F290I)}$ and tor^{XR1}. To generate embryos derived from germ cells doubly homozygous for $Draf^{(R217L,\ F290I)}$ and $Ras1^{\Delta C40B}$, *w* $Draf^{(R217L,\ F290I)}$/*w* $Draf^{(R217L,\ F290I)}$; FRT^{82B} $Ras1^{\Delta C40B}$/*TM3, Sb* females were crossed to $Draf^{(R217L,\ F290I)}$/*Y;* $hsp70\text{-}Flp^{38}$/+; FRT^{82B} $P[ovo^{D1},w^{+}]$/+ males. Following heat-shock induction of the larvae, w^{+}; Sb^{+} females of the genotype: *w* $Draf^{(R217L,\ F290I)}$/*w* $Draf^{(R217L,\ F290I)}$; $hsp70\text{-}FLP^{38}$/+; FRT^{82B} $Ras1^{\Delta C40B}$/FRT^{82B} $P[ovoD1, w^{+}]$, were collected and their eggs which were derived from $Draf^{(R217L,\ F290I)}$ and $Ras1^{\Delta C40B}$ germ cells, examined.

To determine the effect of DrafΔN overexpression on *tll* expression, one copy of the *hsp70-DrafΔN* transgene (Brand and Perrimon, 1994) was introduced from the father by crossing females of the appropriate genotypes with males homozygous for the *hsp70-DrafΔN* transgene. 0- to 1-hour-old embryos were collected on agar plates and allowed to develop for an additional hour at 25°C. They were heat-shocked by floating the plates in a 37°C water bath for 50 minutes, cooled to 4°C, then fixed for in situ hybridization using a *tll* probe.

Production of $Draf\Delta N^{tor4021}$ mRNA and microinjection

A pGEM7 plasmid vector containing $Draf\Delta N^{tor4021}$ sequence (Dickson et al., 1992; gift from Dr L. Ambrosio) was linearized with *Eco*RI and used to produce $Draf\Delta N^{tor4021}$ mRNA using the mMESSAGE mMACHINE SP6 RNA polymerase kit from Ambion (Austin, TX). Synthetic mRNAs were dissolved in nuclease-free distilled water and the concentration determined by UV absorption at 260 nm.

For microinjection, 0- to 1-hour-old embryos were collected and dechorionated with 50% bleach. Early syncytial blastoderm stage embryos were injected at the posterior pole using a pulled glass needle filled with synthetic $Draf\Delta N^{tor4021}$ mRNA. Injection was carried out at 18°C. Injected embryos were allowed to develop for more than 48 hours at 18°C under halocarbon oil. After cuticle development, embryos were washed with heptane, transferred to distilled water containing 0.1% Triton X-100, and then to glycerol:acetic acid (1:3) solution and incubated for 1 hour at 60°C. Cuticle preparations were done according to the method of van der Meer (1977).

Examination of embryos

Digoxigenin-labeled antisense RNA probes were generated using a *tll* cDNA plasmid (Pignoni et al., 1990, 1992). Whole-mount in situ hybridizations were performed according to the method of Tautz and Pfeifle (1989). Embryos were mounted in Euparal (Carolina Biological Supply) following in situ staining or Hoyer's mount (for cuticle preparations).

Physical interactions between Ras1 and Draf or Draf mutants

The Ras1-Draf interaction was examined using the yeast two-hybrid system described by Gyuris et al. (1993). The plasmid containing the fusion of Ras1 sequence encoding amino acid 1 to 185 and the B42 activation domain used in the assay was as described by Hou et al. (1995). The Draf CR1 domain (amino acid 176 to 316) was PCR amplified and fused in frame to LexA in pEG202 vector (Hou et al., 1995). Draf mutant CR1 domains were amplified from genomic DNA isolated from pupae of $Draf^{C110}$, *Su*($Draf^{C110}$)2, and *Su*($Draf^{C110}$)3, respectively.

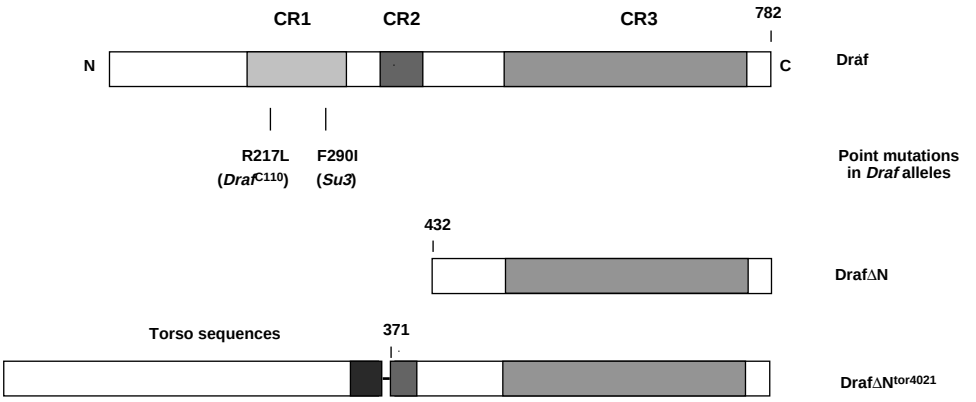

Fig. 1. Schematic representations of wild-type and mutant Draf proteins. The three conserved regions (CR) are shown. The Ras-binding domain (RBD) and cysteine-rich domain (CRD) are located in CR1. CR2 is rich in serine and threonine residues. CR3 contains the serine/threonine kinase domain. The fusion protein DrafΔN^{tor4021} contains Tor extracellular (open bar) and transmembrane (filled bar) domains.

RESULTS

N-terminally truncated Draf requires Ras1 to activate *tll*

Previous data have suggested that N-terminally truncated forms of Raf are 'constitutively active' due to the relief of the N-terminal inhibitory domain (Heidecker et al., 1990; Stanton et al., 1989). However, none of these data were obtained in the complete absence of Ras activity (see Introduction). To examine the effects of total elimination of Ras1 on the signaling properties of 'activated Draf', we expressed a truncated form of Draf (DrafΔN) in embryos entirely lacking maternal *Ras1* activity. DrafΔN encodes a protein in which the N-terminal region (amino acids 2-431), including both the CR1 and CR2 domains, has been deleted (Fig. 1). DrafΔN, or an N-terminally truncated human RAF-1, when introduced into flies as a transgene, has been shown to exhibit activated Draf activities; i.e., heat-shock overexpression of DrafΔN in wild-type animals generates phenotypes reminiscent of gain-of-function Ras1/Draf mediated pathways (Brand and Perrimon, 1994; Casanova et al., 1994; A. Brand, X. Lu and N. Perrimon, unpublished data).

We expressed DrafΔN by heat-shock induction of one copy of a hsp70 promoter-controlled transgene (*hsp70*-DrafΔN; Brand and Perrimon, 1994) in embryos of different genetic backgrounds. This construct was provided paternally to eggs of different maternal genotypes. In wild-type embryos, the posterior domain of *tll* expression extends from the posterior tip to approx. 15% of the egg length anteriorly (Fig. 2A; Pignoni et al., 1992). While *Draf* or *tor* null embryos show no *tll* expression in the posterior region (Fig. 2C, E; Melnick et al., 1993; Pignoni et al., 1992), heat-shock induced DrafΔN was able to expand *tll* expression in wild-type embryos (Fig. 2B; A. Brand, X. Lu and N. Perrimon, unpublished data; see also Casanova et al., 1994), and to rescue to significant levels the posterior *tll* expression in *Draf* null embryos (Fig. 2D). Consistent with previous observations (Casanova et al., 1994; Li et al., 1997), posterior *tll* expression is more sensitive to the level of activated Draf such that ubiquitous expression of activated Draf preferentially activates *tll* in the posterior.

If the posterior domain of *tll* expression, activated by DrafΔN in *Draf* null embryos, was the result of a Ras1-independent constitutive activity of DrafΔN, we would expect that DrafΔN should function similarly in *Ras1* null embryos. Loss of *Ras1* gene activity causes similar but less severe

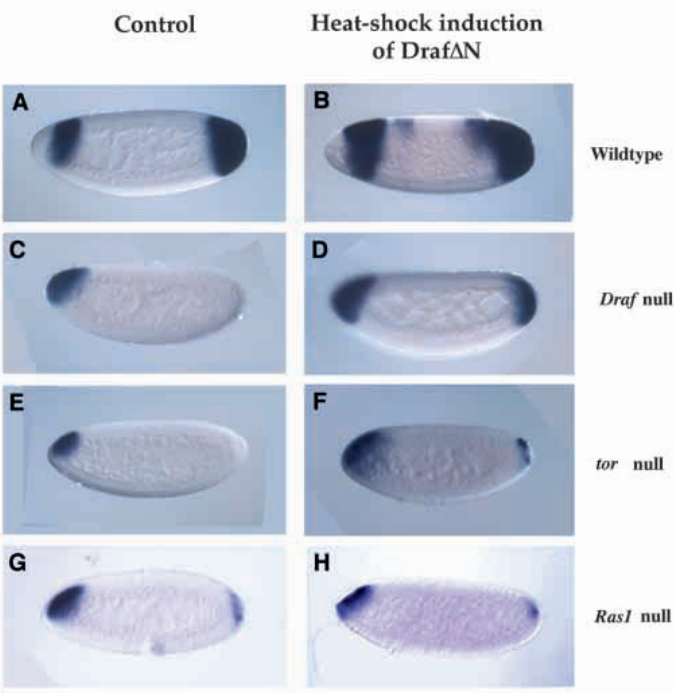

Fig. 2. The biological activity of DrafΔN depends on Ras1. The effects of expression of DrafΔN on *tll* expression levels in early embryos of different genetic backgrounds are shown. Draf activities are reflected by the extent of posterior *tll* expression. Heat-shock induction of a DrafΔN transgene, provided paternally, was able to expand *tll* expression in 23% (n=100) of wild-type embryos (B). Expression of DrafΔN was able to activate posterior *tll* expression to a significant level in 24% (n=86) of *Draf* null embryos (D), while there was an additional 10% that showed residual posterior *tll* expression (not shown). In *tor* null embryos the effect of expressing DrafΔN by the same heat-shock treatment was only marginal, such that following heat-shock induction of DrafΔN, 31% (n=134) of *tor* null embryos showed residual posterior *tll* expression similar to that shown in F, 1.5% (two embryos) showed posterior *tll* expression levels comparable to those shown in D. When expressed in *Ras1* null embryos, no discernible effects on *tll* expression were seen (G and H; n=73). *tll* staining in the anterior region, which is controlled by both Tor and Bicoid pathways (Pignoni et al., 1992), was used as a positive control.

phenotypes than loss of *Draf* activity. Most *Ras1* null embryos are either identical to those of *Draf* or *tor* null embryos, or retain residual levels of posterior *tll* expression in about 20% of them (Fig. 2G; Hou et al., 1995), presumably due to a Ras1-independent pathway that is able to weakly activate Draf in the absence of Ras1 (Hou et al., 1995). Significantly, when DrafΔN was induced in *Ras1* embryos under the same conditions as those used to rescue *Draf* embryos, *tll* expression was not expanded (Fig. 2H). These results suggest that Ras1 is required for the function of DrafΔN. In *tor* null embryos, however, the same level of heat-shock induced DrafΔN resulted in only residual posterior *tll* expression (Fig. 2F), and only occasionally *tll* expression levels similar to those induced in *Draf* embryos were observed (not shown). Because Tor is the upstream RTK that activates Ras1 in terminal development, the differential ability of DrafΔN to activate *tll* expression in *Draf* and *tor* null embryos is consistent with a requirement for activated Ras1 in the signaling potential of DrafΔN. However, since induction of DrafΔN is associated with low levels of posterior *tll* expression in *tor* but not *Ras1* null embryos, we propose that it reflects the presence in *tor* mutants of a small amount of Ras1-GTP (see Discussion).

In summary, when we express DrafΔN at a moderate level, that is nearly sufficient to substitute for the endogenous Draf (Fig. 2D), DrafΔN requires activated-Ras1 to induce the expression of the downstream target gene *tll*.

Membrane-targeted DrafΔN requires Ras1 to activate *tll*

To investigate whether Ras1 is still required for Draf activation once DrafΔN is targeted to the plasma membrane, we used a chimeric protein, DrafΔN^{tor4021}, that is artificially targeted to the membrane through Tor N-terminal sequences. DrafΔN^{tor4021} is a fusion of the signal sequence, extracellular and trans-membrane domains of Tor and a truncated Draf in which the CR1 and part of the CR2 have been removed (Fig. 1; Dickson et al., 1992). The Tor sequence in DrafΔN^{tor4021} contains the point mutation, Y327C, in the extracellular domain that renders the Tor^{4021} protein constitutively active, presumably through ligand-independent dimerization (Sprenger and Nüsslein-Volhard, 1992). Fusion with Tor^{4021} sequences in such a fashion has proved to activate heterologous kinases (Dickson et al., 1992; Groshans et al., 1994), and the same DrafΔN^{tor4021} fusion protein has been shown to be a potent activator of the Tor as well as Sevenless pathways (Baek et al., 1996; Dickson et al., 1992).

It has been demonstrated that different concentrations of DrafΔN^{tor4021} mRNA, when injected into early *Drosophila* embryos, can direct the synthesis of proportionally different amounts of the DrafΔN^{tor4021} fusion protein. At an mRNA concentration as low as 0.001 μg/μl, over 50% of the posteriorly injected *Draf* null embryos can be rescued, as assayed by their abilities to differentiate Filzkörper material (Baek et al., 1996). When we injected DrafΔN^{tor4021} mRNA into early embryos of different mutant backgrounds, markedly different rescuing abilities were observed. When injected into the posterior of *Draf* null embryos, DrafΔN^{tor4021} was highly efficient in rescuing the posterior cuticular structures including the A8 and the Filzkörper (Fig. 3C,D; Baek et al., 1996). In contrast, at each concentration injected, the DrafΔN^{tor4021} mRNA was much less effective in rescuing *Ras1* null embryos. Thus no rescue was observed at 0.01 and 0.1 μg/μl, while at these concentrations about 50% of injected *Draf* null embryos developed Filzkörper (Fig. 3D). At higher concentrations, however, some rescue of *Ras1* null embryos was seen, though the frequencies were lower than *Draf* null embryos. For example, at a concentration of 1 μg/μl DrafΔN^{tor4021} mRNA,

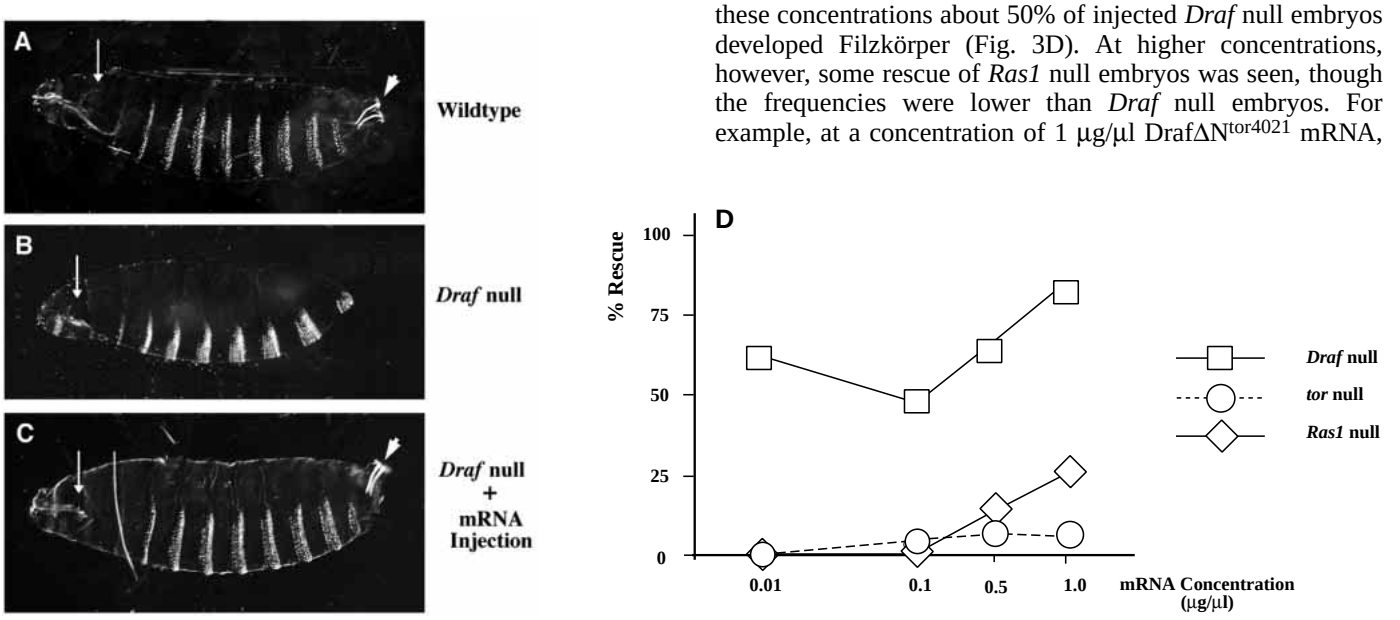

Fig. 3. Membrane-targeted DrafΔN requires Ras1 for activity. mRNA from the fusion gene DrafΔN^{tor4021} (see text for details) was injected at different concentrations into early embryos of different genetic backgrounds. Rescuing ability of injected DrafΔN^{tor4021} was scored by the appearance of Filzkörper material (shown by arrowhead in A and C). Embryos null for *Draf* have collapsed head skeletons (arrow) and are missing all structures posterior to A7, including the Filzkörper (B). When *Draf* null embryos were rescued by injection of DrafΔN^{tor4021} mRNA into the posterior, the Filzkörper developed, whereas the head skeletons, serving as an internal control, remained collapsed (C). D shows percentage rescue of embryos of different genetic background following injection of DrafΔN^{tor4021} mRNA at different concentrations. Without injection, Filzkörper were never observed in *Draf* or *tor* null embryos, but were found in about 1% of *Ras1* null embryos. Usually 200 embryos were injected in each case. Each data point was calculated from at least 50 embryos that had developed cuticles.

26% and 84% of injected *Ras1* null and *Draf* null embryos were rescued, respectively (Fig. 3D). We reason that 1 μg/μl of DrafΔN^{tor4021} mRNA is probably too high a concentration to reflect physiological conditions of the embryo. That is, while injection of 1 μg/μl DrafΔN^{tor4021} mRNA solution results in expression of the fusion protein at levels similar to those of the endogenous Draf (Baek et al., 1996), all the protein is membrane localized and concentrated in the posterior region, whereas under wild-type conditions only a small amount of Raf becomes membrane localized following RTK activation (Hallberg et al., 1994).

When DrafΔN^{tor4021} mRNA was injected into *tor*^{XR1} embryos (XR1 is a protein null allele), about 5% of injected embryos were rescued at each concentration except at 0.01 μg/μl, where no rescue was observed. Interestingly, unlike in *Ras1* null embryos, the rescuing ability of DrafΔN^{tor4021} mRNA in *tor*^{XR1} embryos was not significantly increased when the injection concentration was raised to 1 μg/μl (Fig. 3D). The simplest explanation to accommodate these results is that the activity of the fusion protein DrafΔN^{tor4021} requires Ras1 in its GTP-bound state, which is abundant at the termini of *Draf* null embryos due to the presence of the activated Tor, while possibly the presence of only a small amount of Ras1-GTP in *tor* null embryos allows occasional activation of DrafΔN^{tor4021} (see Discussion).

Therefore, contrary to previous models, the membrane-targeted DrafΔN^{tor4021} requires Ras1 for its activity. We envision two alternative models to account for this observation. First, in addition to recruiting Draf to the membrane, Ras1 is also required for Draf activation. This second Ras1-dependent requirement cannot be bypassed by simply placing Draf at the membrane. Second, the different effects of exogenously expressed modified forms of Draf in *Draf*, *tor* and *Ras1* embryos could reflect the absence of wild-type Draf protein in *Draf*, but not *tor* and *Ras1* null embryos. Possibly, inactive, endogenous wild-type Draf could act as a dominant-negative inhibitor of the chimeric Draf protein by competing for a factor that is limiting in amount.

Intragenic suppressors of *Draf*^{C110} do not restore Ras1:Draf interaction

To distinguish between the two models discussed above, we examined the signaling activity of a mutant Draf protein that has lost its Ras1-binding ability but yet retained the ability to mediate Tor signaling. Previously, we isolated a hypomorphic mutation *Draf*^{C110}, R217L, that prevented its binding to Ras1 (Perrimon et al., 1985; Hou et al., 1995; Melnick et al., 1993). The *Draf*^{C110} mutant allele, associated with reduced Draf activity, is still able to mediate normal Tor signaling. *Draf*^{C110} animals die before emerging from their pupal cases probably due to insufficient zygotic Draf activity during larval development. Indeed these animals develop eye defects reminiscent of a loss of RTK activity in the eye (Melnick et al., 1993; Lu et al., 1994), and this phenotype is more severe over a deficiency of the region. Although the Draf^{C110}:Ras1 interaction is abolished, we wanted to determine whether Ras1 still plays a role in the function of Draf^{C110}. To test this would require examining embryos from *Draf*^{C110}; *Ras1* double mutant germ lines, which is not technically feasible because the mutations are on different chromosomes. To overcome this problem, we used an intragenic suppressor of *Draf*^{C110}, *Su3*, that effectively rescues the lethality associated with *Draf*^{C110} (Lu et al., 1994).

Two dominant, intragenic suppressors of *Draf*^{C110}, *Su2* and *Su3*, are caused by second site mutations, P308L and F290I, respectively, located within the CRD Ras binding domain within the CR1 region (Fig. 1; Lu et al., 1994). Animals doubly mutant for *Draf*^{C110} and *Su3* or *Su2* are 98% and 18% viable over *Draf*^{C110}, respectively (Lu et al., 1994). When mutations equivalent to *Draf*^{C110} and either one of its intragenic suppressors were introduced into the human RAF-1 protein, it was found that the intragenic suppressor mutations do not restore the Raf:Ras interaction or increase the affinity of Raf-1 to Ras (Cutler and Morrison, 1997). We used the yeast two-hybrid system (Fields and Song, 1989; Gyuris et al., 1993) to confirm that this is also true when the *Draf*^{C110}, along with either *Su3* or *Su2*, were introduced into the *Drosophila* proteins. While we were able to detect interaction between the wild-type CR1 fragment of Draf with Ras1 (Fig. 4; also Hou et al., 1995), the mutant CR1 fragment from *Draf*^{C110} was unable to interact with Ras1, as measured by β-galactosidase

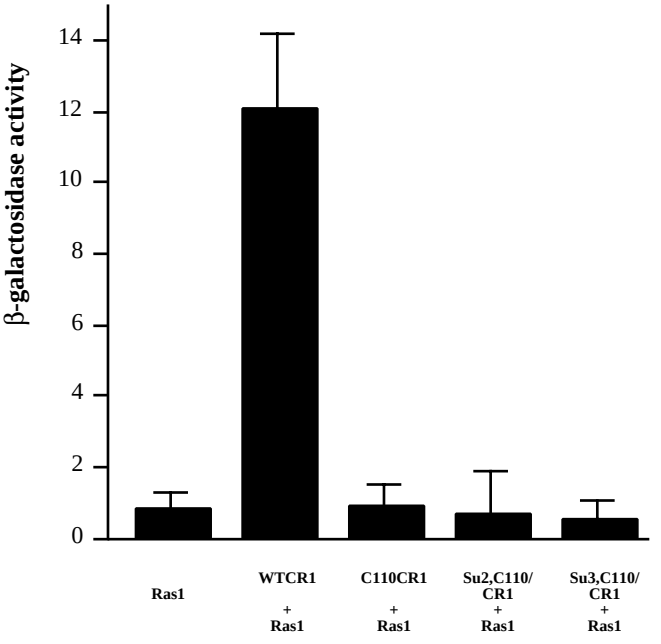

Fig. 4. Effect of the *Draf*^{C110} mutation and its intragenic suppressors on binding of Ras1 to Draf. The yeast two hybrid system (Gyuris et al., 1993) was used to demonstrate the affinity of Ras1 for the Draf CR1 domain, and the disruption of this interaction when the *Draf*^{C110} mutation (R217L) was introduced. Affinity between the proteins is reflected in the transcription of β-galactosidase, whose activity is given in units as defined by Rose et al. (1990). When the *Draf*^{C110} mutation is introduced, β-galactosidase activity is reduced to a background level similar to that with Ras1 alone. When CR1 fragments doubly mutant for the *Draf*^{C110} mutation and its intragenic suppressors, *Su2* and *Su3*, respectively, were used in this assay, no elevation of β-galactosidase activity was detected, indicating that these suppressors do not restore the Ras1-Draf association. The expression levels of Ras1 and the Draf CR1 fragment were equal in these experiments, as measured by western blotting (data not shown). Error bars indicate the standard deviations between the results of a minimum of four independent yeast colonies assayed for each experiment and control.

activity, which was at a similar level to control cells transfected with Ras1 alone (Fig. 4; see also Hou et al., 1995). When mutant CR1 fragments harboring the original *Draf*^{C110} mutation in combination with *Su2* or *Su3*, respectively, was co-transfected with Ras1 into yeast cells, no increase in the Ras1:CR1(*Su2*) or Ras1:CR1(*Su3*) interactions were detected (Fig. 4). Therefore, consistent with the results obtained with the human RAF-1 protein, two *Drosophila* intragenic suppressors, *Su2* and *Su3*, in combination with the *Draf*^{C110} mutation were unable to physically interact with Ras1. These results suggest that the restoration of Draf function to the *Draf*^{C110} mutant by these suppressors is due to mechanisms that do not involve the physical interaction between Ras1 and the mutant Draf. The viability of the *Draf*^{C110}/*Su3* double mutation allowed us to evaluate the in vivo regulation of a mutant Draf protein, Draf$^{(R217L,\ F290I)}$, that does not bind to Ras1.

Draf$^{(R217L,\ F290I)}$ is regulated by Ras1

Studies with mutations in the human RAF-1 have shown that the intragenic suppressor mutations, including those equivalent to the *Drosophila Su2* and *Su3*, in combination with the human *DRAF*^{C110} equivalent mutation, R89L, have increased basal biological, as well as enzymatic activities that are not affected by exogenously introduced Ras. Cutler and Morrison (1997) have suggested that the suppressors confer constitutive activities on the original mutant Raf protein which could explain the viability of the *Drosophila* double mutants. To investigate whether Draf$^{(R217L,\ F290I)}$ is able to function in embryos lacking maternal Ras1, we examined embryos from homozygous *Draf*$^{(R217L,\ F290I)}$ females bearing mosaic germline cells homozygous for a protein null *Ras1* allele.

Due to the presence of the Ras1-independent pathway that can weakly activate Draf in the absence of Ras1, residual posterior *tll* expression can be detected in about 20% of *Ras1* null embryos, while the remaining 80% of the embryos show no *tll* expression (Hou et al., 1995; Li et al., 1997). Consistent with their *tll* expression levels, the larval cuticles of *Ras1* null embryos show more posterior structures than those from either *tor*^{XR1} or *Draf* null embryos (Hou et al., 1995). While the majority of *Ras1* null embryos have only seven abdominal denticle bands, similar to those of *tor*^{XR1} or *Draf* null embryos, about 20% of them retain A8, and about 1% of these embryos have Filzkörper material. Animals homozygous for *Draf*$^{(R217L,\ F290I)}$ alone are viable, morphologically normal, and their larval cuticles as well as *tll* expression levels are indistinguishable from those of wild-type embryos (Fig. 5A,B). However, all embryos derived from *Draf*$^{(R217L,\ F290I)}$; *Ras1* germ cells die during embryogenesis and develop cuticles indistinguishable from those of embryos mutant for *Ras1* alone (Fig. 5H,J). Posterior *tll* expression in embryos doubly mutant for *Ras1* and *Draf*$^{(R217L,\ F290I)}$ extends slightly more anteriorly than those of *Ras1* null embryos (Fig. 5G,I). The modest anterior extension of *tll* expression may reflect an additive effect of the Ras1-independent activation and the unregulated activities of Draf$^{(R217L,\ F290I)}$, as were detected in assays with the mutant human RAF-1.

In summary, the phenotypes of *Draf*$^{(R217L,\ F290I)}$; *Ras1* double mutant embryos resemble those from *Ras1* alone rather than wild type, suggesting that Draf$^{(R217L,\ F290I)}$ is unable to mediate Tor signaling in the absence of *Ras1* gene activity.

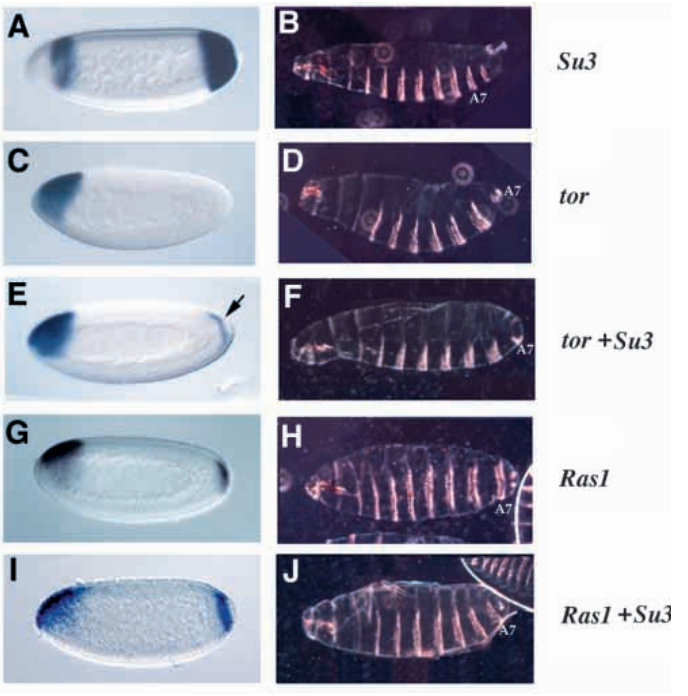

Fig. 5. Draf$^{(R217L,\ F290I)}$ is regulated by Ras1 and Tor. *tll* expression levels (A,C,E,G and I) and larval cuticles (B,D,F,H and J) are shown. Embryos derived from female germ lines homozygous for both *Draf*$^{(R217L,\ F290I)}$ and *Ras1*$^{\Delta C40B}$ (I and J) are more similar to those homozygous for *Ras1*$^{\Delta C40B}$ alone (G and H) than to *Draf*$^{(R217L,\ F290I)}$ homozygotes (A and B), which are indistinguishable from wild type. Embryos from females doubly mutant for *Draf*$^{(R217L,\ F290I)}$ and *tor*^{XR1} (E and F) showed *tll* expression and cuticles identical to those from females homozygous for *tor*^{XR1} alone (C and D), except that 20% of the embryos showed residual posterior *tll* expression (arrow in E) and develop cuticular structures posterior to the seventh abdominal denticle band, A7 (F). Note in *tor*^{XR1} embryos A7 is at the posterior tip, whereas in 20% of *Draf*$^{(R217L,\ F290I)}$; *tor*^{XR1} double mutants A7 is in a more anterior position.

Therefore, Ras1 still regulates a mutant Draf, Draf$^{(R217L,\ F290I)}$, that lacks the ability to physically interact with Ras1. These results favor the model that a Ras1-dependent activity is required for Draf activation independent of its membrane translocation.

The activity of Draf$^{(R217L,\ F290I)}$ is controlled by Tor

To determine to what extent Draf$^{(R217L,\ F290I)}$ is regulated by Tor and to what extent its activity is constitutive, we generated animals doubly mutant for *Draf*$^{(R217L,\ F290I)}$ and the *tor*^{XR1} protein null allele. Embryos from *tor*^{XR1} females lack posterior *tll* expression and the larval cuticles are missing all structures posterior to the seventh abdominal segment (A7; Fig. 5C,D). Embryos derived from females doubly mutant for *Draf*$^{(R217L,\ F290I)}$ and *tor*^{XR1} were completely non-viable. Their cuticle phenotypes and levels of *tll* expression are almost identical to those of embryos from *tor*^{XR1} females, although about 20% of these embryos exhibit residual posterior *tll* expression (Fig. 5E) as well as slightly more differentiated cuticular structures posterior to A7 than those from *tor*^{XR1} mothers (Fig. 5F). The residual *tll* expression indicates that Draf$^{(R217L,\ F290I)}$ retains low level activity that is not controlled

by Tor, consistent with studies with human RAF-1 mutations, where the *Draf*^{C110} equivalent mutation, R89L, in combination with any of the suppressor mutations exhibits increased basal level activities. Our in vivo results in *Drosophila* extend the vertebrate results by demonstrating that the mutant Draf$^{(R217L, F290I)}$ protein is still regulated by Tor and that its constitutive activity is sufficiently low such that the mutant protein alone is unable to effect normal Tor signaling.

DISCUSSION

We have used the early *Drosophila* embryo as an in vivo system to investigate the requirement for Ras in Raf activation. Using the 'FLP-DFS' technique, we were able to examine the activity of various Draf proteins in embryos that completely lack Ras1 activity. Such a situation has not been possible in mammalian cell lines because Ras activity is required for cell viability. We have found that two activated forms of Draf (one N-terminally truncated and the other N-terminally truncated as well as membrane targeted), when expressed at moderate levels, are not active in the absence of Ras1. Further, we demonstrate that a mutant Draf protein, Draf$^{(R217L, F290I)}$, that has lost its Ras1-binding ability is functional, yet its activity requires input from Ras1. To explain these results, we propose that, in addition to translocating Raf to the plasma membrane, Ras-GTP also activates an unknown 'Raf activator'.

The role of Ras-Raf association in the activation of Raf

The finding that Ras1 is required for the activation of mutant Draf proteins that do not physically interact with Ras1, does not undermine the importance of the Ras-Raf association in Raf activation in wild type. It has been proposed that the association between Ras and Raf accomplishes two functions. First, it facilitates Raf activation by translocating Raf to the plasma membrane. Second, this association also relieves the inhibitory effects of the N-terminal region of Raf (Drugan et al., 1996; Morrison and Cutler, 1997). The CRD in the CR1 region of Raf is the second Ras-binding domain that makes contact with Ras through a different set of residues than those that interact with RBD, the first Ras-binding domain. CRD will not interact with Ras unless the RBD-Ras interaction has occurred (Drugan et al., 1996; Morrison and Cutler, 1997), suggesting that Ras, through sequential binding to the two domains, induces a conformational change in Raf. Such an event may cause a dissociation of the inhibitory N terminus from the C terminus, which would be readily accessible to the 'Raf activator'.

In *Drosophila*, the *Draf*^{C110} point mutation, R217L, abolishes the molecular interaction between Ras1 and Draf, and results in a defective protein that cannot support the survival of the animal. Because the *Draf*^{C110} mutation retains Tor signaling activity, it suggests that recruitment of Raf to the membrane by Ras is dispensable under certain circumstances. Perhaps Raf can reach the membrane by passive diffusion. The two intragenic suppressors, *Su2* and *Su3*, which restore sufficient Raf activity to rescue the lethality associated with *Draf*^{C110}, are located in the CRD of Draf, yet do not restore the Ras1:Draf interaction. Possibly, these suppressor mutations disrupt the association between the N- and C-terminal halves of Draf, resulting in a more 'open' configuration of the protein. Such modifications may be analogous to the conformational change that results from the binding of Ras to Raf. Therefore, it is possible that the Draf$^{(R217L, F290I)}$ protein has acquired an altered conformation, which is more accessible to the 'Raf activator' than the Draf^{C110} mutant protein.

Ras is required for Raf activation in addition to membrane translocation of Raf

Ras is located at the inner surface of the plasma membrane and cycles in two states: the active GTP-bound form and the inactive GDP-bound form. Much of our knowledge about the function of Ras in Raf activation has relied on studies using proteins expressed in tissue culture cells. The conclusion that the sole function of Ras in Raf activation is to translocate Raf to the plasma membrane was mainly drawn from co-transfection experiments demonstrating that the membrane-targeted Raf is activated and cannot be inhibited by Ras^{N17}, a dominant-negative form of Ras that competes with wild-type Ras for binding to the guanine-nucleotide exchange factor (GEF; Farnsworth and Feig, 1991; Leevers et al., 1994; Stokoe et al., 1994). It is conceivable that some residual endogenous Ras activity was still present in these experiments as the dominant-negative Ras protein used may not completely inhibit wild-type Ras function. This possibility would be consistent with studies in *Drosophila*, which have indicated that injection of Ras^{N17} into wild-type embryos is unable to generate phenotypes more severe than those observed in *Ras1* null embryos (Lu et al., 1993; Hou et al., 1995).

Without extracellular stimuli, tissue culture cells are perhaps analogous to *tor* null embryos. We found that *tor* null embryos are able to support the activation of an N-terminally truncated or membrane-targeted Draf, though to levels lower than in *Draf* null embryos, in which the endogenous Tor is activated by its ligands. We propose that the lower activity levels exhibited by the modified Draf proteins in *tor* null embryos are due to the presence of only a minority of GTP-bound Ras1 in the absence of the upstream RTK Tor, while the bulk of Ras is in its GDP-bound form. A similar hypothesis has been proposed in the study of the function of a *Drosophila* 14-3-3 protein in Tor signaling (Li et al., 1997). Overexpression of 14-3-3 results in activation of Draf in wild-type, but not in *Ras1* null embryos, suggesting that Ras1 is required for 14-3-3-induced activation of Draf. However, low levels of Draf activation are observed in *tor* null mutant embryos when 14-3-3 is overexpressed. We proposed that the activation of Draf by 14-3-3 in *tor* null embryos reflects the presence of a small amount of Ras1-GTP present in the embryo, which is in equilibrium with the majority of Ras1-GDP (Li et al., 1997). However, if expressed at non-physiologically high levels, DrafΔN is likely to exhibit significantly higher levels of activity, as it has previously been shown that heat-shock overexpression of DrafΔN can rescue the posterior defects of *tor* null embryos (Casanova et al., 1994; A. Brand, X. Lu and N. Perrimon, unpublished data).

It could be argued that the inability of truncated forms of Draf to activate the Tor pathway in *Ras1* and *tor* mutant backgrounds is due to competition with endogenous, inactive Draf that is associated with a protein present in limiting quantity. This model is unlikely for the following reasons. First, in wild-type tissue culture cells, only 3% of Raf-1 translocates to the membrane and becomes activated as a result

of RTK activation (Hallberg et al., 1994). Thus, the overwhelming majority of Raf-1 is inactive and yet does not compete with the activated Raf-1. Second, it could be argued that wild-type Raf and the truncated Draf compete for a limited pool of the Draf substrate, MEK. However, this is unlikely since MEK is probably activated once it is bound to Raf (Huang et al., 1993). Finally, the requirement for Ras1 in the activation of Draf$^{(R217L,\ F290I)}$ argues against the presence of a limited amount of Raf-associated factor whose function is independent of Ras, because Draf$^{(R217L,\ F290I)}$ would be constitutively active in a *Ras1* null background if such a factor were Ras-independent.

An alternative model to explain our finding that activated Raf requires Ras1 activity is to postulate that Ras1 activates a Draf-independent pathway which, in combination with Draf, is required to induce *tll* expression. There is no evidence for such a pathway since *tll* is not expressed in the absence of Draf and weaker *Draf* alleles exhibit reduced levels of *tll* expression (Melnick et al., 1993; Hou et al., 1995; Li et al., 1997). Further, the phenotype associated with *Draf*$^{(R217L,\ F290I)}$ also argues against the existence of such a Draf independent pathway from Ras1 to *tll*. In *Draf*$^{(R217L,\ F290I)}$; *Ras1* double mutant embryos *tll* expression is very reduced. We reason that if this effect reflected the down-regulation of only a Draf-independent pathway then it would imply that *Draf*$^{(R217L,\ F290I)}$ is not regulated by Ras1 and therefore can only be constitutively active, because low activity of Draf is associated with lethality and reduced *tll* expression. This does not appear to be the case because *Draf*$^{(R217L,\ F290I)}$ does not exhibit any dominant phenotypes in a wild-type background. Therefore, the simplest explanation for our results is that Ras1 is required to activate Draf in addition to recruiting it to the membrane. Such an interpretation is consistent with recent reports of Mineo et al. (1997) and Roy et al. (1997). Mineo et al., (1997) identified a mutant Ras protein that binds to a mutant, but not wild-type Raf-1. When the mutant Raf-1 protein is targeted to the membrane, it can be additionally activated by interacting with this mutated form of Ras, suggesting that the Ras/Raf-1 interaction plays a role in Raf-1 activation that is distinct from membrane recruitment. Further, Roy et al. (1997) generated an activated Raf-1 by replacing two tyrosine residues with aspartic acid (RafDD) and found the activity of membrane-localized RafDD can be additionally stimulated by Ras. This activation is abolished by a mutation in the zinc finger located within the CRD of RafDD, suggesting that Ras activates Raf-1 through its zinc finger.

In summary, we have shown that mutant or modified Draf proteins that have lost their Ras-binding abilities are capable of signaling, and that their activities are dependent on Ras1 in its active form. The role of Ras1 in the activation of these mutant Draf proteins is not in the membrane translocation of the Draf, as membrane targeting of a truncated Draf does not bypass the requirement for Ras1. Therefore, we conclude that activated Ras is required in Raf activation beyond the membrane-translocation step.

Dual function of Ras in Raf activation

Currently, the exact mechanism by which Raf is regulated is still unknown. Besides Ras, other Raf-interacting proteins have been isolated as potential Raf activators. These include 14-3-3 (reviewed by Aitken, 1995; Morrison, 1994) and KSR (Kornfeld et al., 1995; Sundaram and Han, 1995; Therrien et al., 1995). Studies with the *Drosophila* 14-3-3 genes indicate that they cannot encode the 'Raf activator' since these proteins are necessary, but not sufficient for Draf signaling (Chang and Rubin, 1997; Kockel et al., 1997; Li et al., 1997). The situation with KSR is less clear. KSR associates with 14-3-3 and also with Raf-1 at the membrane in a Ras-dependent manner (Therrien et al., 1996; Xing et al., 1997). Zhang et al. (1997) have suggested that KSR is a ceramide-activated protein (CAP) kinase that phosphorylates and activates Raf-1 in vitro. However, Therrien et al. (1996) and Yu et al. (1997) found that KSR plays a structural role in modulating Raf/MEK/MAPK signal propagation and it does not appear to phosphorylate Raf-1.

Besides these possible Raf activators, it has also been shown that artificial oligomerization can activate Raf-1, presumably as a result of conformational change or transphosphorylation or both (Farrar et al., 1996; Luo et al., 1996). However, the effect of forced oligomerization on Raf-1 activation appears much weaker than that stimulated by the activation of an upstream RTK, and it is not clear whether Raf activation requires oligomerization in vivo (Farrar et al., 1996; Luo et al., 1996; reviewed by Morrison and Cutler, 1997). The activity of the fusion protein DrafΔN^{tor4021}, especially at high concentrations, may therefore result from homodimerization of itself as well as heterodimerization with the endogenous Tor, as they share the same extracellular and transmembrane domains. Heterodimerization between DrafΔN^{tor4021} and Tor could cause tyrosine phosphorylation and activation of the former, as it has been shown that tyrosine phosphorylation of Raf-1 by Src can activate Raf-1 (Marais et al., 1995). Such activation may be Ras1-independent, as expression of DrafΔN^{tor4021} following injection of high concentrations of mRNA is capable of restoring Tor signaling in *Ras1* null embryos.

Finally, it is possible that very high concentrations of DrafΔN^{tor4021} mRNA are capable of restoring Tor signaling in *Ras1* null embryos because of the activity of the Ras1-independent pathway that activates Draf (Hou et al., 1995). In the absence of Ras1, residual *tll* expression still remains in the posterior of the embryo, indicating that there is Ras1-independent Draf activation. The nature of this activity is unclear. It could originate from a pathway regulated by Tor and thus operates in parallel to the Ras1 pathway. Alternatively, it could be due to a factor that is activated only when Ras1 is removed from the Tor pathway. Perhaps in the absence of Ras1 another small GTPase, such as Ras2, can replace Ras 1. Regardless of the nature of this Ras1-independent pathway, it is not able to replace the function of Ras1 in activating the 'Draf activator' (Fig. 6) at physiological levels of Draf. However, perhaps when non-physiologically high levels of Draf are provided, this parallel activity can complement for lack of Ras1 (see experiment in Fig. 3D).

Model of Raf activation

In this study, we have provided evidence that mutant or truncated Draf proteins that do not bind to Ras1 still require Ras1 for activation. Assuming that the yeast two hybrid assay is an accurate method to detect the physical interaction that occurs between Ras and Raf molecules in vivo, our findings, together with studies in mammalian cultured cells, support a

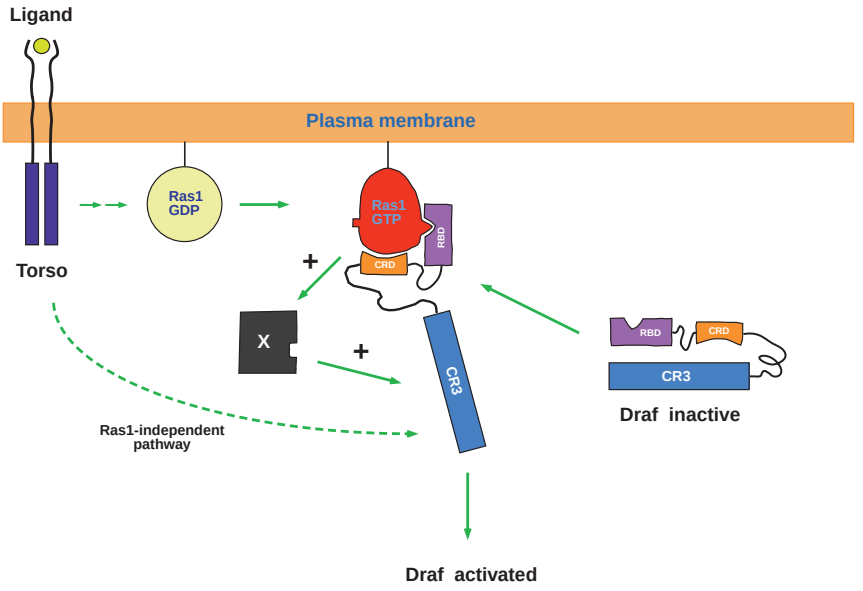

Fig. 6. Model for the role of Ras1 in Draf activation. (1) GTP-Ras interacts directly with Raf at the membrane. This physical association induces a conformational change in Raf and also places Raf in close proximity to the uncharacterized 'Raf activator', indicated by 'X'. (2) Independent of the Ras-Raf association, GTP-Ras is required for the activation the 'Raf activator', which in turn contributes to the activation of Raf. See text for more details.

'two-step' mechanism (Fig. 6) by which Ras activates Raf. First, we propose that activated Ras binds to Raf at the membrane and brings Raf into close proximity to its activator. The conformational change that results from the binding of Ras to the N-terminal region of Raf relieves the inhibitory effects of the N terminus, exposing the CR3 kinase domain to the 'Raf activator'. Since it has been shown that Ras-GTP does not directly activate Raf, the existence of a 'Raf activator' has to be postulated. Second, we propose that, independent of the binding of Ras to Raf, activated Ras activates the unknown 'Raf activator'. A test of this model obviously awaits the identification of the 'Raf activator'.

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