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The Torso Receptor Tyrosine Kinase Can Activate Raf in a Ras-Independent Pathway

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Summary

Activation of the receptor tyrosine kinase (RTK) torso defines the spatial domains of expression of the transcription factors *tailless* and *huckebein*. Previous analyses have demonstrated that Ras1 ($p21^{ras}$) operates upstream of the D-Raf (Raf1) serine/threonine kinase in this signaling pathway. By using a recently developed technique of germline mosaics, we find that D-Raf can be activated by torso in the complete absence of Ras1. This result is supported by analysis of D-Raf activation in the absence of either the exchange factor Son of sevenless (Sos) or the adaptor protein drk (Grb2), as well as by the phenotype of a *D-Raf* mutation that abolishes binding of Ras1 to D-Raf. Our study provides in vivo evidence that Raf can be activated by an RTK in a Ras-independent pathway.

Introduction

Studies on receptor tyrosine kinase (RTK) signaling pathways in both vertebrates and invertebrates have converged on an evolutionarily conserved cassette of genes that are required for transducing the signal from the membrane to the nucleus (reviewed by Egan and Weinberg, 1993; Perrimon and Desplan, 1994; Dickson and Hafen, 1994). The serine/threonine protein kinase Raf occupies a central role in this pathway. When Raf becomes activated in response to RTK activation, it phosphorylates the tyrosine/threonine kinase MEK, which in turn phosphorylates the serine/threonine kinase MAPK. Subsequently, through phosphorylation, MAPK modifies the activity of a subset of transcription factors. The mechanism of Raf activation is still unresolved (reviewed by Morrison, 1994; Daum et al., 1994). Studies in both mammalian cells and invertebrate systems have implicated $p21^{ras}$ as a positive regulator of Raf (reviewed by Perrimon and Desplan, 1994). Indeed, the GTP-bound form of $p21^{ras}$ has been found to bind directly to the CR1 domain of Raf (Vojtek et al., 1993; Moodie et al., 1993). However, this association does not lead to Raf activation but appears to promote the translocation of Raf to the membrane in which it subsequently becomes activated by an unknown mechanism (Stokoe et al., 1994; Leevers et al., 1994). In addition to binding $p21^{ras}$, Raf molecules, both cytosolic and membrane-bound forms, are also associated with the 14-3-3 proteins. It has been speculated that these proteins play a role in Raf activation; however, the function of the 14-3-3 proteins remains unclear (reviewed by Morrison, 1994). Furthermore, it is not known whether activation of Raf at the membrane requires additional input from the RTK (reviewed by Daum et al., 1994).

The mechanism by which RTKs control $p21^{ras}$ activation is better understood (reviewed by Egan and Weinberg, 1993; Perrimon and Desplan, 1994). Following ligand binding, the RTK dimerizes, which triggers transphosphorylation of the receptor on tyrosine residues (reviewed by van der Geer et al., 1994). These phosphotyrosines in the cytoplasmic domain of the RTK serve as docking sites for various proteins, one of which is Grb2, also known as SEM-5 in Caenorhabditis elegans (Clark et al., 1992) and downstream of receptor kinases (drk) in Drosophila (Olivier et al., 1993; Simon et al., 1993), which contains one SH2 and two SH3 domains. Through its interaction with the Grb2 SH3 domains, the $p21^{ras}$-exchange factor Son of sevenless (Sos) translocates to the membrane where it promotes the exchange of $p21^{ras}$–GDP to $p21^{ras}$–GTP. Also involved in the regulation of $p21^{ras}$ are Ras–Gap enzymes, which increase the endogenous Ras–GTPase activity (reviewed by McCormick, 1993).

To determine precisely the contribution of $p21^{ras}$, Sos, Ras–Gap, and Grb2 to Raf activation, we have examined the effect on Raf activation of removing any one of these gene activities. We have assayed the role of these genes in the Drosophila torso (tor) RTK signaling pathway, which is involved in defining terminal embryonic cell fates (reviewed by Duffy and Perrimon, 1994). Tor is the first RTK pathway that becomes activated in the Drosophila embryo. Tor RTK is expressed uniformly in the egg (Casanova and Struhl, 1989) and becomes activated locally in the syncitial blastoderm at both poles in response to an activity localized in the perivitelline space (Sprenger and Nusslein-Volhard, 1992; Casanova and Struhl, 1993). Activated tor triggers the Raf/MEK/MAPK phosphorylation cascade (reviewed by Duffy and Perrimon, 1994) that ultimately leads to the localized expression of the transcription factors *tailless* (*tll*; Pignoni et al., 1990, 1992) and *huckebein* (*hkb*; Weigel et al., 1990; Brönner et al., 1994) at the termini of the embryo. In the wild-type cellular blastoderm, the posterior domains of expression of *tll* and *hkb* overlap, and their expression is solely dependent upon the tor signaling pathway. *tll* is expressed in the 0%–15% egg length (EL) interval, and *hkb* is expressed in the 0%–8% EL interval. The differences between these two posterior domains of expression reflect the differential responses of the *tll* and *hkb* promotors to the strength of the tor signaling pathway, since no other patterning systems repress the posterior expression of these genes before the blastoderm stage (reviewed by Perkins and Perrimon, 1991). Thus, the spatial domain of *tll* and *hkb* expression can be used as a readout for the strength of the tor signal transduction cascade. An increase in tor signaling, as observed in the case

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of *tor* gain-of-function mutations, is associated with an expansion of *tll* expression toward the middle of the embryo (Steingrimsson et al., 1991). A decrease in tor signaling is associated with a retraction of *tll* and *hkb* expression toward the embryonic termini (Casanova and Struhl, 1989).

Similarly, the posterior domains of expression of *tll* and *hkb* are an accurate measure of the state of D-Raf activation. *tll* and *hkb* are not expressed posteriorly in the complete absence of D-Raf activity (Ambrosio et al., 1989a; Pignoni et al., 1992; Lu et al., 1993). Mutations in *D-Raf* that have residual activity are associated with a retraction of *tll* and *hkb* expression toward the embryonic termini (Melnick et al., 1993). Finally, expression of activated forms of D-Raf in embryos are associated with a phenotype reminiscent of the *tor* gain-of-function mutations (Casanova et al., 1994; A. Brand, X. Lu, and N. P., unpublished data).

Previous analyses have implicated a role for the *Ras1* ($p21^{ras}$), *Sos*, *Gap1* (*Ras–Gap*), and *drk* (*Grb2*) genes in tor signaling (Lu et al., 1993; Doyle and Bishop, 1993). However, their precise roles have not been examined in detail. These genes are associated with zygotic lethality, reflecting their functions in multiple RTK signaling pathways (Perrimon, 1994). To analyze the function of these molecules in tor signaling, we have generated germline mosaics of null mutations in these genes. Here, we report that D-Raf is activated by the tor RTK in the absence of Ras1, a finding supported by the phenotype of embryos lacking either Sos or drk activity, as well as by the phenotype of a *D-Raf* mutation that abolishes binding of Ras1 to D-Raf.

Results

Analyses of the Effects of Ras1, Sos, Gap1, and Drk on Terminal Development

Mutations in *D-Raf*, *Ras1*, *Sos*, *Gap1*, and *drk* are associated with zygotic lethality (Perrimon et al., 1985; Rogge et al., 1991; Simon et al., 1991; Gaul et al., 1992; Simon et al., 1993; Olivier et al., 1993). Since the tor terminal system is deposited maternally (reviewed by Duffy and Perrimon, 1994), a direct way to examine the role of these essential genes in tor signaling is to examine the development of eggs derived from mosaic females that have a homozygous mutant germline. To generate germline mosaics, we used the FLP-DFS technique (Chou and Perrimon, 1992; Chou et al., 1993; T.-B. C. and N. P., unpublished data), which allows the efficient production of females with germline clones (see the Experimental Procedures for details).

D-Raf Can Be Activated by Tor without Ras1

All embryos derived from females homozygous for a null *tor* mutation (tor^{XR1}) that does not produce the tor protein (Sprenger and Nusslein-Volhard, 1992; Sprenger et al., 1993) exhibit terminal defects that include all structures posterior to the seventh abdominal segment and a collapsed head skeleton. These defects correlate with altered expression patterns of the transcription factors *tll* and *hkb* (Pignoni et al., 1992; Weigel et al., 1990; Lu et al., 1993). These gap genes are not expressed posteriorly in *tor* embryos. Anteriorly, *tll* and *hkb* are expressed in response to the additional regulatory input from the bicoid system (Pignoni et al., 1992; Ronchi et al., 1993). However, these anterior expression patterns are abnormal, with *tll* expanded and *hkb* reduced.

Loss of maternal *D-Raf* activity has effects similar to tor on the regulation of *tll* and *hkb* (see Figures 1B1 and 1B2). However, the cuticle phenotypes of the embryos that develop vary depending upon the paternal contribution (Perrimon et al., 1985; Ambrosio et al., 1989a; Melnick et al., 1993). If *D-Raf* mutant embryos have received a wild-type copy of the *D-Raf* gene from their fathers (*D-Raf*-rescued embryos), they develop cuticle that resembles that of *tor* embryos (see Figure 3B). However, if they have not received a copy of wild-type *D-Raf* (*D-Raf*-null embryos), they only differentiate remnants of cuticle with no obvious pattern (see Figure 3C). The differences between *D-Raf*-null and *D-Raf*-rescued embryos reflect the role of this kinase in multiple RTK signaling pathways. There are no differences between the expression patterns of *hkb* and *tll* in null versus rescued *D-Raf* embryos (see Experimental Procedures). In *D-Raf*-rescued embryos, the only known signaling pathway affected is tor, while in *D-Raf*-null embryos, signaling from tor, as well as zygotic RTKs such as DER (Drosophila epidermal growth factor receptor), is blocked (Melnick et al., 1993).

To determine the role of Ras1 in tor signaling, we examined the phenotypes of embryos derived from *Ras1* mutant germlines. If, as predicted by recent models of Raf activation (Stokoe et al., 1994; Leevers et al., 1994), Raf becomes activated at the membrane following its Ras-mediated translocation, then we expect *Ras1* embryos to

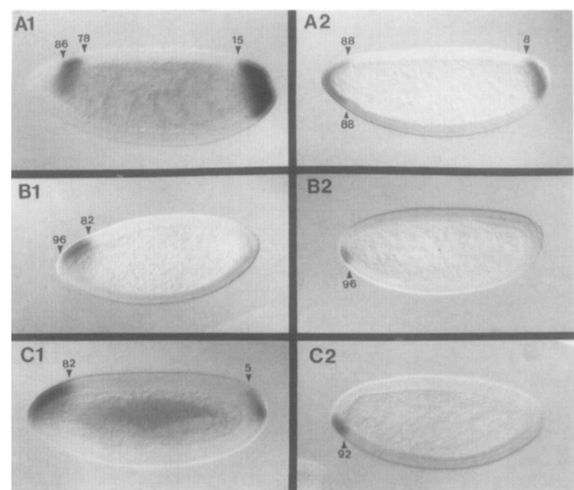

Figure 1. Tor Activates D-Raf in a Ras1-Independent Pathway

The in situ hybridization patterns of *tll* (A1, B1, and C1) and *hkb* (A2, B2, and C2) are shown in wild-type (A), $D\text{-}Raf^{11-29}$ (B), and $Ras1^{\Delta C40b}$ (C) embryos. Note that *tll* is expressed posteriorly in $Ras1^{\Delta C40b}$ embryos but not in $D\text{-}Raf^{11-29}$ embryos. All embryos are oriented with the anterior to the left and dorsal up. The domains of *tll* and *hkb* expression are indicated as percent egg length, with 0% corresponding to the posterior pole.

Table 1. Molecular Properties of the Mutations Used in This Study

Mutation	Lesion	References
D-Raf$^{11-29}$	Protein null	Sprenger et al. (1993)
Ras1$^{\Delta C40b}$	Deletion of *Ras1*	This study
Sos^{e4G}	Nonsense in residue 421	Simon et al. (1991)
drk$^{\Delta P24}$	Deletion of *drk*	T. Raabe and E. Hafen (personal communication)
Gap1^{B2}	Genetic null	Gaul et al. (1992)

Sos^{e4G} most likely represents a complete loss of function, since it is associated with a termination codon at amino acid position 421 that deletes the drk-binding site as well as the catalytic domain (Simon et al., 1991, 1993; Bonfini et al., 1992; Olivier et al., 1993). It is not known whether *Sos*^{e4g} makes a truncated protein.

have a phenotype identical to *tor* or *D-Raf* mutants with respect to both *tll* and *hkb* expression. We produced females with germlines completely lacking Ras1 protein (Table 1; Figure 2; Experimental Procedures) and analyzed the embryonic development of the resulting embryos. There are no differences between the expression patterns of *hkb* and *tll* in null versus rescued *Ras1* embryos. In *Ras1* embryos, the posterior expression pattern of *tll* is reduced to 5% EL at the blastoderm stage; anteriorly, *tll* expression is expanded (Figure 1C1). This result indicates that tor signaling is not, as observed in *D-Raf* mutants (Figure 1B1), completely blocked by removal of the *Ras1* gene. This observation is consistent with the expression of *tll* in wild-type embryos injected with a dominant negative form of Ras (Lu et al., 1993). The effect on *tll* expression in *Ras1* embryos correlates with the pattern of *hkb* expression (Figure 1C2). Posteriorly, *hkb* is not expressed in *Ras1* mutants, suggesting that the *hkb* promotor is more sensitive to a reduction in tor signaling than the *tll* promotor. Anteriorly, *hkb* expression is reduced less than in either *tor* or *D-Raf* mutants (compare Figures 1C2 and 1B2), again indicating that tor signaling is not completely blocked in *Ras1* mutants.

The effect of lack of *Ras1* activity on the establishment of terminal cell fates is also evident when the cuticle phenotypes of *Ras1* embryos are examined. Unlike *tor*- and *D-Raf*-rescued mutants (Figure 3B), *Ras1*-rescued embryos differentiate some structures posterior to A7 (A8 and in some cases the posterior spiracles; Figures 3D and 3E). The presence of these structures in *Ras1* mutants is consistent with the domain of *tll* expression at the blastoderm stage (see Perkins and Perrimon, 1991, for a fate map of the terminalia). In addition, *Ras1*-null embryos develop poorly but appear to differentiate slightly more cuticular structures than *D-Raf*-null embryos (compare Figures 3F and 3C). This result indicates that signaling not only from tor but also from other RTKs is not completely blocked in *Ras1*-null embryos.

Interestingly, *Ras1* mutant embryos show defects in segmentation that are not observed in *D-Raf* or *tor* mutants. A number of segmental fusions are observed (Figure 3G), which are already apparent at the blastoderm stage (Figure 3H) as detected by abnormal expression of the pair-rule gene *fushi tarazu* (*ftz*). Since these segmentation defects are not observed in *D-Raf* embryos, it indicates that *Ras1* is involved in developmental pathways that do not use the D-Raf kinase. This result is not unexpected, since Ras has downstream targets other than Raf (reviewed by Feig and Schaffhausen, 1994).

A D-Raf Mutant Protein That Abolishes Binding of Ras1 to D-Raf Can Activate Tailless

The observation that C-Raf1 binds Ras via its CR1 domain (Vojtek et al., 1993) led us to examine the effect of a *D-Raf* mutation within the CR1 domain, *D-Raf*^{C110}, on the Ras1/D-Raf association. *D-Raf*^{C110} is associated with the amino acid change Arg-217 to Leu (Melnick et al., 1993). This change reduces D-Raf activity, since *D-Raf*^{C110} behaves genetically as a hypomorphic mutation (Perrimon et al., 1985). Interestingly, *tll* and *hkb* expression are not affected

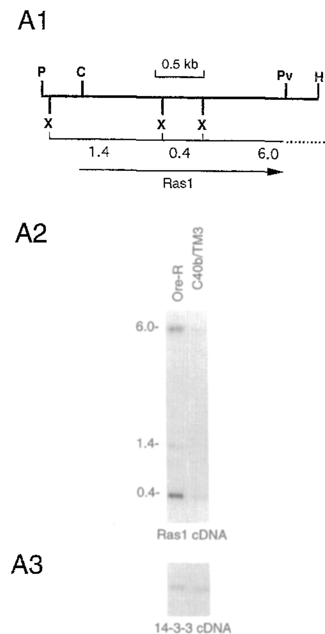

Figure 2. *Ras1*$^{\Delta C40b}$ Is a Deletion of the *Ras1* Gene

(A1) Restriction map of the *Ras1* gene.

(A2) To detect the nature of the lesions associated with *Ras1* mutations, equal amounts of DNA from heterozygous flies were digested with XmnI, blotted onto nitrocellulose, and probed with the entire *Ras1* cDNA. The restriction fragments that are missing in *Ras1*$^{\Delta C40b}$ have an intensity half as great as those from the homozygous Oregon-R (Ore-R) control DNA, indicating that *Ras1*$^{\Delta C40b}$ is a deletion of the *Ras1* gene.

(A3) shows the same Southern blot hybridized with a *D-14-3-3* cDNA probe (Swanson and Ganguly, 1992) to quantitate the amounts of DNA present. Abbreviations: C, ClaI; H, HindIII; P, PstI; Pv, PvuII; X, XmnI.

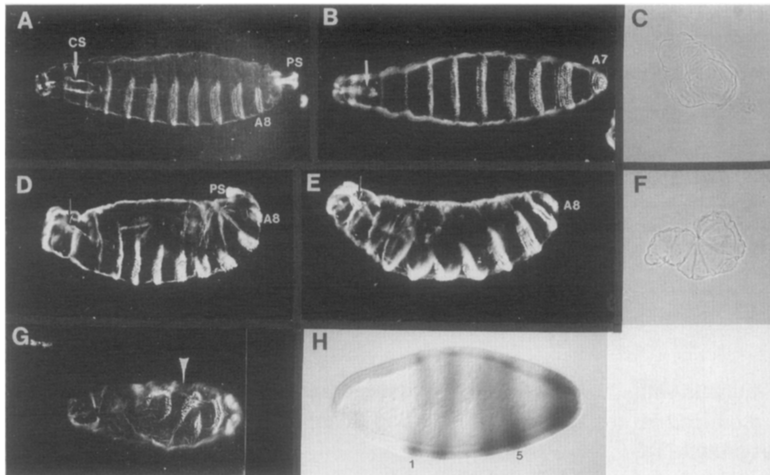

Figure 3. *Ras1* Embryos Develop More Cuticular Elements Than *D-Raf* Embryos

The cuticle phenotypes of wild-type (A), $D\text{-}Raf^{11\text{-}29}$-rescued (B), $D\text{-}Raf^{11\text{-}29}$-null (C), $Ras1^{\Delta C40b}$-rescued (D, E, and G), and $Ras1^{\Delta C40b}$-null (F) embryos are shown. Both $D\text{-}Raf^{11\text{-}29}$- and $Ras1^{\Delta C40b}$-null embryos develop poorly (C and F). Note that $D\text{-}Raf^{11\text{-}29}$-rescued embryos are missing all structures posterior to the seventh abdominal segment, while $Ras1^{\Delta C40b}$-rescued embryos develop more posterior structures (i.e., posterior spiracles [D] and the eighth abdominal segment [D and E]). Both $D\text{-}Raf^{11\text{-}29}$- and $Ras1^{\Delta C40b}$-rescued embryos have defects in the cephalopharyngeal skeleton (arrows in [B], [D], and [E] compared with wild type in [A]). Of $Ras1^{\Delta C40b}$-rescued embryos, 50% show abdominal segmentation defects that are not seen in $D\text{-}Raf^{11\text{-}29}$-rescued embryos. Abnormal segmentation of the abdominal region of $Ras1^{\Delta C40b}$ embryos can be detected at the blastoderm stage. (H) shows the abnormal expression of *ftz* in a $Ras1^{\Delta C40b}$-rescued embryo (of genotype $Ras1^{\Delta C40b}$/*TM3, Sb, ftz–lacZ*). Note that the third stripe of *ftz* expression is eliminated in this embryo, the sixth stripe is expanded toward the posterior, and the seventh stripe is deleted as observed in terminal class mutants (Ambrosio et al., 1989a; Lu et al., 1993). Abbreviations: ps, posterior spiracles; A7 and A8 are the abdominal segments 7 and 8; cs, cephalopharyngeal skeleton.

in $D\text{-}Raf^{C110}$ embryos (Melnick et al., 1993; data not shown). To test whether this mutation affects the interaction between Ras1 and D-Raf, we utilized the yeast two-hybrid system (Gyuris et al., 1993). We were able to reproduce the Ras/Raf interaction using the fly molecules and to show that the $D\text{-}Raf^{C110}$ mutation abolishes any interaction between Ras1 and D-Raf (see Experimental Procedures). Mutation of the corresponding amino acid residue in C-Raf1 (Arg-89) has confirmed this result (Fabian et al., 1994). Thus, consistent with the analysis of *Ras1* mutants, a mutation in D-Raf that prevents the binding of Ras1 to D-Raf can still transduce the signal from tor. This provides further evidence that D-Raf can be activated by tor in the absence of Ras1. Interestingly, the $D\text{-}Raf^{C110}$ mutant phenotype is not as severe as the *Ras1* mutant phenotype, suggesting that apart from its effect on the Ras1 interaction the $D\text{-}Raf^{C110}$ change may also weakly activate D-Raf (see Discussion).

The Role of Gap1 and Sos in Tor Signaling

The activity of Ras1 is regulated by two enzymes, Gap1 and Sos (McCormick, 1993). Gap1 encodes a Ras–Gap protein (Gaul et al., 1992) that acts as a negative regulator of Ras1, presumably by promoting the conversion of Ras1–GTP to Ras1–GDP. Sos is a positive regulator of Ras1 and encodes a nucleotide exchange factor (Rogge et al., 1991; Simon et al., 1991; Bonfini et al., 1992) that promotes the conversion of Ras–GDP to Ras–GTP. To determine the requirement of these enzymes in tor signaling, we examined the phenotypes of embryos derived from germline clones of both *Gap1* and *Sos* mutations.

Embryos derived from germline clones homozygous for the genetic null *Gap1* allele, $Gap1^{B2}$ (Gaul et al., 1992), were examined for *tll* and *hkb* expression. At the cellular blastoderm stage, the domains of *tll* and *hkb* expression are clearly expanded toward the center of the embryo (Figures 4C and 4D), indicating that in wild-type animals Gap1 acts as a negative regulator of tor signaling. Interestingly, loss of *Gap1* activity only expands *tll* up to its original domain of expression. In wild-type precellular embryos, *tll* is initially expressed in the 0%–20% EL interval and then quickly retracts by the blastoderm stage to 0%–15% (Pignoni et al., 1990, 1992). While the initial domain of *tll* expression in *Gap1* precellular embryos is not different from wild-type (data not shown), *tll* in *Gap1* cellular blastoderm embryos does not retract to 0%–15% (Figure 4C). Thus, removal of *Gap1* activity does not expand the domain of

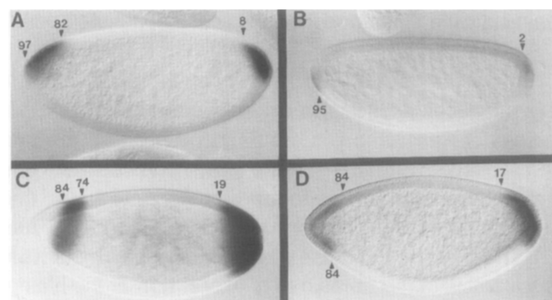

Figure 4. Roles of the Ras1 Regulators, Sos and Gap1, in Tor Signaling

(A) and (B) show the expression patterns of *tll* and *hkb*, respectively, in Sos^{e4G} embryos. Note that *tll* expression is reduced in Sos^{e4G} embryos, as observed in the case of $Ras1^{\Delta C40b}$ embryos. However, 20% of Sos^{e4G} embryos have residual posterior *hkb* expression that is never detected in $Ras1^{\Delta C40b}$ embryos.

(C) and (D) show *tll* and hkb expression in $Gap1^{B2}$ embryos. Most (95%) of the $Gap1^{B2}$ embryos develop a wild-type cuticle (data not shown; see also Chou et al., 1993). The modest expansion of *tll* expression may explain why the cuticle pattern of *Gap1*-null or *Gap1*-rescued embryos is not severely affected (data not shown). The remaining embryos exhibit variable segmentation defects including defects in dorsoventral patterning. These embryos are probably derived from egg chambers that possess both *Gap1* homozygous germline and follicle cell clones. Defects in dorsoventral patterning reflect the follicle cell function of Gap1 downstream of the EGF receptor (Chou et al., 1993; Brand and Perrimon, 1994).

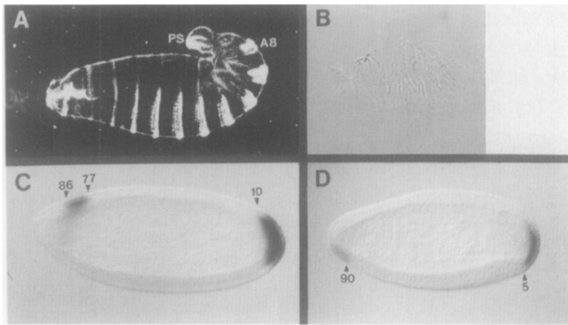

Figure 5. Removal of *drk* Activity Has a Weaker Effect on Embryonic Development Than Removal of Either *Sos*, *Ras1*, or *D-Raf* Activities

The cuticle phenotypes of *drk*$^{\Delta P24}$-rescued (A) and *drk*$^{\Delta P24}$-null (B) embryos are shown. More cuticle elements are present in *drk*$^{\Delta P24}$ than in either *Sos*^{e4G} or *Ras1*$^{\Delta C40b}$ mutant animals. (C) and (D) show the expression patterns of *tll* and *hkb*, respectively, in *drk*$^{\Delta P24}$ embryos. The domain of *tll* expression in *drk*$^{\Delta P24}$ embryos is more extensive than the domain of *tll* expression in either *Sos*^{e4G} or *Ras1*$^{\Delta C40b}$ embryos. In addition, substantial *hkb* expression is detected in *drk*$^{\Delta P24}$ embryos.

tll expression per se, but results in an up-regulation of Ras1 activity within a domain that is initially defined by activated tor.

Germline clone analysis of a genetic null allele, *Sos*^{e4G} (Table 1), indicates that Sos acts positively in tor signaling (see also Lu et al., 1993; Doyle and Bishop, 1993). In *Sos* embryos (Figure 4), the domains of expression of both *tll* and *hkb* are similar to the expression patterns of these genes in *Ras1* embryos. However, loss of Sos activity is associated with a less severe phenotype than the complete loss of *Ras1*. At the posterior, *tll* is expressed in the 0%–8% EL interval in *Sos* mutants (Figure 4A) compared with 5% in *Ras1* mutants (see Figure 1C1). *hkb*, which is never expressed at the posterior of *Ras1* mutants, is expressed in a small posterior domain (Figure 4B) in 20% of the *Sos* embryos examined. There are no differences between the expression patterns of *hkb* and *tll* in null versus rescued *Sos* embryos. Differences between *Ras1* and *Sos* embryonic phenotypes are also apparent when the cuticles of *Ras1* and *Sos* embryos are compared (data not shown). While *Ras1*-rescued animals rarely differentiate filzkorper material and posterior spiracles, *Sos*^{e4G}-rescued embryos have some posterior spiracle materials and a partial A8. Similarly, *Sos*-null mutants differentiate more cuticular elements than *Ras1*-null mutants.

Removal of Drk Activity Has a Weaker Effect Than Removal of Either Sos or Ras1 Activity

Drk encodes the homolog of Grb2/SEM-5 and acts as an adaptor between a phosphotyrosine of the activated RTK and Sos (Olivier et al., 1993; Simon et al., 1993). To determine the role of drk in tor signaling, we examined the embryonic phenotype of eggs derived from germlines that are homozygous for a deletion of the *drk* gene (*drk*$^{\Delta P24}$; Table 1; T. Raabe and E. Hafen, personal communication). In *drk* embryos, the domains of expression of both *tll* and *hkb* are reduced from wild type, indicating that drk acts positively in tor signaling. However, the effect of loss of *drk* activity is not as severe as removing either *Sos* or *Ras1*. *tll* in *drk* embryos is expressed in the 0%–10% EL interval, and *hkb* is expressed between 0%–5% EL (Figures 5C and 5D). There are no differences between the expression patterns of *hkb* and *tll* in null versus rescued *drk* embryos. Differences between *drk* and *Sos* embryonic phenotypes are also obvious when the cuticular embryonic phenotypes are examined. In *drk*-null embryos, a significant amount of cuticle differentiation can be detected (Figure 5B). In *drk*-rescued animals, defects in the posterior spiracles and A8, which are common in *Sos* animals, are rarely observed (Figure 5A).

Discussion

We have used germline mosaics to analyze the respective contribution of the *Ras1*, *drk*, *Sos*, and *Gap1* genes to tor signaling. Since these molecules are not required for cell proliferation of the germline, we can analyze the contribution of each of these components to tor signaling. Our results demonstrate that D-Raf is activated in the absence of Ras1, thus providing direct evidence of a Ras1-independent pathway that activates D-Raf. We also demonstrate that the activation of Ras1 does not follow a simple linear pathway, since removal of drk does not provide a phenotype identical to the removal of Sos and removal of Sos is not identical to the removal of Ras1.

Activation of Raf by a Ras1-Independent Pathway

Our results indicate that, in the absence of Ras1 activity, *tll* is activated posteriorly and that this domain of expression is spatially reduced. Since D-Raf acts downstream of Ras1, and in the absence of D-Raf or tor activity *tll* is not expressed posteriorly, our results demonstrate that tor is able to activate D-Raf using a Ras1-independent pathway. The activation of D-Raf by the Ras1-independent pathway is regulated by tor itself and does not reflect the presence of a nonregulated D-Raf activation system. This is demonstrated by the observation that in *tor* mutants *tll* is not expressed posteriorly (Pignoni et al., 1992; Lu et al., 1993). In addition, the localized expression of *tll* in *Ras1* embryos does not reflect a spatial restriction in the ability of *tll* to become activated. This is evident from the uniform *tll* expression in embryos derived from females that express a constitutively activated form of tor (Steingrimsson et al., 1991), a phenotype that is completely suppressed when D-Raf activity is removed (Ambrosio et al., 1989b).

Could the Ras1-independent activation of D-Raf by tor reflect a phenomenon specific to the mutant cell? Possibly, removal of Ras1 protein from the early embryo could lead to the activation of a novel pathway that activates D-Raf. Alternatively, in the wild-type animal, Ras1 could actively suppress the Ras1-independent activation of D-Raf. Our analysis of the *D-Raf*^{C110} mutation, which affects the binding of Ras1 to D-Raf, argues against such models. We find that some level of D-Raf activation occurs not only when embryos develop in the complete absence of Ras1 protein, but also when wild-type Ras1 is unable to bind D-Raf due to the *D-Raf*^{C110} mutation. In addition, the exis-

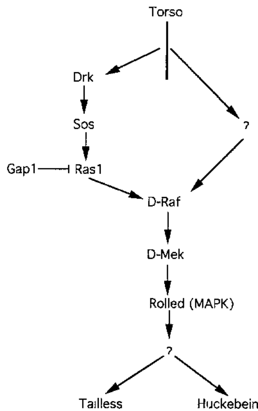

Figure 6. Model of D-Raf Activation

tence of a Ras1-independent pathway is consistent with results obtained from injections of a dominant negative form of Ras, p21^{rasN17}, in wild-type embryos that only partially blocks tor signaling (Lu et al., 1993).

Our results suggest that in wild-type animals full activation of D-Raf requires activities transduced along two pathways both regulated by tor, a Ras1-dependent pathway that involves drk, Sos, Gap1, and Ras1 and a Ras1-independent pathway (Figure 6). The mechanisms by which these two pathways cooperate to provide full D-Raf activation are not yet clear. In one model, the sole function of Ras1 is to regulate the level of D-Raf available at the membrane (see also Stokoe et al., 1994; Leevers et al., 1994) where the Ras1-independent pathway subsequently activates D-Raf. Our results are consistent with this model, but in addition demonstrate that Ras1 is not absolutely required for this activation process. D-Raf may be translocated to the membrane in the absence of Ras1, whereby the Ras1-independent pathway may activate a sufficient amount of D-Raf to allow activation of the MEK/MAPK pathway. Alternatively, cytoplasmic D-Raf could become activated from an activity regulated by tor. In a second model, both the Ras1-dependent and Ras1-independent pathways could independently activate D-Raf to some extent. Synergism between two weakly activating pathways could lead to full activation of D-Raf. Consistent with this model is the observation that activated forms of Ras can turn on *tll* and *hkb* in *tor* mutant embryos (Lu et al., 1993). Distinguishing between these two models will have to await the identification of mutations in components of the Ras1-independent pathway.

Nature of the Ras1-Independent Pathway

What is the nature of the Ras1-independent pathway? The observation that D-Raf can become activated in the absence of Ras1 could reflect redundancy at the level of the *Ras* genes. To date, three Drosophila *Ras* genes that belong to different *Ras* gene families have been isolated: *Ras1*, *Ras2*, and *Ras3* (reviewed by Lev, 1993). *Ras1* belongs to the Ras family, which includes the three human transforming *Ras* genes; *Ras2* belongs to the family that includes *R-ras*; and *Ras3* is most similar to the Rap gene family. R-Ras proteins have recently been shown to be able to bind Raf1 (Spaargaren et al., 1994), raising the possibility that Ras2 could partially substitute for Ras1 in D-Raf activation. A number of lines of evidence, however, suggest that it is unlikely. First, R-Ras proteins do not appear to be regulated by the exchange factor Sos (Buday and Downward, 1993). Second, expression of an activated Ras2 protein in the eye does not lead to the production of extra R7 photoreceptor cells, as observed in the case of expression of activated Ras1 (Fortini et al., 1992). Similarly, expression of activated Ras2 in early embryos does not affect terminal cell fate differentiation as oberved in the case of activated Ras1 (Lu et al., 1993). Ras3 is even more unlikely than Ras2 to substitute for Ras1 in D-Raf activation, since it appears to play a negative role in RTK signaling (Hariharan et al., 1991). In conclusion, we favor the existence of a Ras1-independent pathway that regulates D-Raf activity to explain our observation that D-Raf mutants are more severe than Ras1 mutants. Perhaps the most convincing argument in favor of this hypothesis is the observation that even if another *Ras* gene were able to partially suppress the *Ras1* mutant phenotype, it would not explain why *drk* or *Sos* mutants have a phenotype less severe than D-Raf.

The factor that activates Raf may be a Raf kinase kinase (reviewed by Daum et al., 1994). A non-RTK such as Src may be involved in this activating process. Williams et al. (1992) showed that full activation of Raf in insect cells could be induced by a synergistic effect of both Src and Ras. Serine/threonine kinases such as protein kinase C (PKC) may also be directly involved. PKC translocates to the membrane along with Raf upon receptor activation and has been shown to be able to activate Raf-1 by direct phosphorylation both in vitro and in vivo (Kolch et al., 1993; Caroll and May, 1994). Consistent with the idea that Raf requires additional inputs for activation, studies of Raf–CAAX mutants have indicated that Raf activity is low unless Raf becomes further stimulated by a Ras-independent signal (Stokoe et al., 1994; Leevers et al., 1994). Finally, the recently characterized 14-3-3 proteins, which appear to behave as chaperones for Raf, may play a role in the Raf activation process; however, their function(s) still remains obscure (reviewed by Morrison, 1994). Further characterization of this pathway will be required to identify molecules involved in the Ras1-independent pathway.

Signaling Properties Associated with *D-Raf^{C110}*

Analysis of the *D-Raf^{C110}* mutation supports our findings that D-Raf does not absolutely require Ras1 for activation. In the *D-Raf^{C110}* mutation, in which detectable Ras1 binding to D-Raf is abolished, we postulate that the residual D-Raf activation that we detect reflects the function of the Ras1-independent pathway. Relevant to this hypothesis is an analysis of suppressors of *D-Raf^{C110}* (Lu et al., 1994). The strongest of these suppressors, Su3, is an intragenic mutation in the cysteine-finger motif within the C-terminus of the CR1 domain. Using the yeast two-hybrid system, we have found that Su3 does not restore the interaction between Ras1 and D-Raf (data not shown). The motif in which Su3 falls may represent a distinct ligand-binding domain

in D-Raf. It is possible that a decreased affinity for Ras1 may be compensated for by an increased affinity for a member of the Ras1-independent pathway. Candidate Raf interactors are the 14-3-3 proteins, which bind to the CR1 domain as well as to more C-terminal residues (Freed et al., 1994). In addition to four intragenic suppressors, Lu et al. (1994) found six autosomal second-site suppressors, one or more of which may represent activating mutations in components of the Ras1-independent pathway.

The observation that the *D-Raf*^{C110} phenotype is associated with a less extreme phenotype than Ras1 mutants suggests *D-Raf*^{C110} may have additional activities. Possibly, the *D-Raf*^{C110} mutation might both block binding to Ras1 and partially activate the kinase domain. Examination of *tll* expression in embryos double mutant for both *tor* and *D-Raf*^{C110} should help to resolve this issue. The *D-Raf*^{C110} change may alter the D-Raf conformation to make it more open, which has been postulated as being significant for Raf activation (Bruder et al., 1992). In this context, it is intriguing that two of the intragenic suppressors of *D-Raf*^{C110} characterized by Lu et al. (1994) were found in the C-terminal CR3 or kinase domain of D-Raf, consistent with a model in which there is interaction between the N- and C-terminal halves of Raf enzyme. However, no evidence of such an interaction could be found using the two-hybrid system (data not shown).

The Encumbrance Model

Embryos that lack *Sos* activity exhibit a phenotype similar to, but distinctly weaker than, *Ras1* embryos. Since in these experiments we use complete loss-of-function *Ras1* and *Sos* alleles, this result cannot be attributed to residual activity from any of these mutations. We envision two possible explanations for this result. First, other as of yet unidentified exchange factor activities may lead to a low level of Ras1 activation. Second, the absence of Sos in the receptor complex may lead to an up-regulation of the Ras1-independent pathway. Depleting the receptor complex of proteins that play a role in signaling may increase the accessibility of molecules to the Ras1-independent pathway. Biochemical studies have revealed that Raf activation occurs following recruitment of molecules to a receptor complex (reviewed by van der Geer et al., 1994). The proximity of proteins in the receptor complex may affect the kinetics of interactions between some of the components. Thus, in *Ras1* mutants, the Ras1-independent pathway may not be activated to a level comparable to its level of activation in an *Sos*-null mutant because the Sos protein is encumbering the receptor. Similarly, loss of drk activity leads to a reduction in *tll* expression that is weaker than that due to removal of Sos. To explain these effects, we can propose either that Sos can be recruited to the membrane using other unidentified adaptors or, as proposed for Sos, removal of drk from the receptor complex may allow the Ras1-independent pathway to be up-regulated to a level higher than in the presence of drk and absence of Sos. A prediction of this encumbrance hypothesis is that the mutant phenotype of *Sos*- or *drk*-null mutations that produce inactive proteins still able to interact with their partners may be more extreme than the mutant phenotype associated with protein null *Sos* alleles.

Experimental Procedures

Production of Germline Mosaics Using the FLP-DFS Technique

D-Raf, *Ras1*, *Sos*, *drk*, and *Gap1* mutations described in the text are listed in Table 1. Germline clones of the X-linked protein null *D-Raf*$^{11-29}$ allele (Ambrosio et al., 1989b; Sprenger et al., 1993) were generated as described by Melnick et al. (1993). Germline clones of the autosomal mutations (*m*), *Ras1*, *Sos*, *drk*, and *Gap1* were generated using the autosomal-FLP-DFS technique (T.-B. C. and N. P., unpublished data). In brief, females of genotype *CyO/FRT m* or *TM3, Sb/FRT m* were crossed with males of genotype *FLP*^{12}*/Y; CyO*/P[*ovo*^{D1}] *FRT* or *FLP*^{22}/*Y; TM3, Sb*/P[*ovo*^{D1}] *FRT*. Progeny were heat shocked for 2 hr at 37°C during larval stages, and females of genotypes *FLP*^{12}/+; *FRT m*/P[*ovo*^{D1}] *FRT* or *FLP*^{22}/+; *FRT m*/P[*ovo*^{D1}] *FRT* were analyzed for the presence of germline clones. Approximately 90% of mosaic females can be recovered following this heat shock treatment. In each experiment, at least 100 embryos were examined.

A detailed description of the strains used for this analysis will be provided elsewhere. *FLP*^{12} and *FLP*^{22} are two different X-linked flipase insertions. The *FRT* insertions used for each chromosomal arm are the following: *FRT*$^{2L-40A}$ (40A); *FRT*$^{2R-G13}$ (42B); *FRT*$^{3L-2A}$ (79D-F); and *FRT*$^{3R-82B}$ (82B). All of the P[*ovo*^{D1}] *FRT* recombinant chromosomes are associated with a fully penetrant dominant female sterility phenotype (Chou et al., 1993) such that all eggs laid by these females are derived from germ cells that have undergone a mitotic exchange event.

Distinction between Null and Paternally Rescued Embryos

Mosaic females possessing germline clones of a specific autosomal mutation were crossed with males carrying the same mutation over a balancer chromosome that contains a *lacZ* gene. The *lacZ* gene is under the control of either the *hunchback* (*hb*) promotor (*CyO, hb–lacZ*) or the *fushi tarazu* (*ftz*) promotor (*TM3, Sb, ftz–lacZ*). *D-Raf* mosaic females were crossed with *FM7, ftz–lacZ/Y* males. The genotype of embryos was determined by following the expression pattern of the *lacZ* gene. The RNA expression pattern of *lacZ* was detected rather than β-galactosidase activity because it was necessary in our experiments to identify the genotype of the embryos precisely at the blastoderm stage (β-galactosidase activity from these lines does not express well at the blastoderm stage). Embryos without the *lacZ* marker are referred to as "null embryos", since they lack both maternal and zygotic copies of the wild-type gene. Their siblings, which express the *lacZ* gene, are referred to as the "rescued embryos", since they lack only the maternal gene.

We did not detect any difference between the expression patterns of *hkb* and *tll* in null versus rescued *D-Raf*, *Ras1*, *Sos*, *drk*, and *Gap1* embryos. Thus, when discussing the effect of removal of specific gene activities on the expression of these genes, we do not distinguish between the two classes and simply refer to the two classes as "mutant embryos". However, there are obvious cuticular differences between the rescued versus null *Ras1*, *Sos*, and *drk* embryos. These effects are reminiscent of the differences previously observed in the case of *D-Raf* mutations (Perrimon et al., 1985; Ambrosio et al., 1989a; Melnick et al., 1993) and reflect the role of these genes in other zygotic RTK pathways (Melnick et al., 1993). To establish unambiguously the cuticular phenotypes associated with each genotype, we compared the phenotypes of embryos derived from germline clone females crossed either with wild-type (+/+) males or heterozygous (+/*m*) males.

Examination of Embryos

In situ hybridizations on whole-mount embryos using digoxigenin-labeled probes were performed according to Tautz and Pfeifle (1989). Single-stranded sense and antisense digoxigenin-containing DNA probes were prepared by the PCR labeling technique (N. Patel, personal communication) using appropriate primers (Biolabs). Probes were prepared from plasmids containing the following genes: *tll* cDNA (Pignoni et al., 1990, 1992); *hkb* cDNA (Weigel et al., 1990); *Ras1* cDNA (Neuman-Silberberg et al., 1984); *lacZ* coding region (Thummel et al., 1988). For visualization, embryos were dehydrated through an

ethanol series and mounted in Euparal (Carolina Biological Supply). Embryos were analyzed and photographed on a Zeiss Axiophot microscope with Nomarski optics. When double in situ stainings were carried out, embryos were incubated simultaneously with the two probes.

Larval cuticles were prepared in Hoyer's mountant as described by van der Meer (1977). Cuticles were examined using dark-field or phase illumination.

Identification of a Ras1 Protein Null Mutation

Twelve different *Ras1* mutations were obtained from M. Simon, J. Schnorr, and C. Berg. To identify a protein null allele, Southern blots of all *Ras1* mutations were performed. One of them, $Ras1^{\Delta C40b}$, is a complete deletion of the *Ras1* locus (Figure 2). *Ras1* is uniformly expressed in wild-type embryos. No signal is detected in $Ras1^{\Delta C40b}$ blastoderm embryos (data not shown). Probe DNAs were ^{32}P-labeled using the random priming method (Feinberg and Vogelstein, 1983). Southern blot analyses were done as described in Sambrook et al. (1989).

Physical Interaction between Ras1 and D-Raf

The yeast two-hybrid system described by Gyuris et al. (1993) was used to examine the interaction between Ras1 and D-Raf. DNA corresponding to amino acids 1–185 of *Ras1* was cloned into vector JG4-5 to give an in-frame fusion with the B42 activation domain. The last four amino acids (186–189) were removed to eliminate the possibility of Ras1 membrane localization via the CAAX box interfering with the interaction assay.

Initially, DNA corresponding to D-Raf amino acids 1–316 was cloned into vector pEG202 to produce the corresponding lexA fusion protein. However, this molecule did not show significant interaction with Ras1, possibly because D-Raf is about 100 amino acids longer at its N-terminus than the vertebrate Raf molecules. We next made a lexA fusion to the D-Raf CR1 domain alone, namely amino acids 176–316. A PCR product with 5′ EcoRI and 3′ NcoI sites was cloned into the corresponding sites of pEG202. This PCR was performed on both wild-type *D-Raf* and $D\text{-}Raf^{C110}$ genomic DNA isolated as described (Melnick et al., 1993).

These constructs were transformed into yeast cells as described by Gietz et al. (1992), and the interaction between the resultant fusion proteins was assayed as described by Gyuris et al. (1993). For each control and experiment, at least four independent yeast colonies were assayed and standard deviations calculated. β-galactosidase activity, measured in units defined by Rose et al. (1990), reflects the affinity between the molecules tested. Expression of Ras1 and D-Raf CR1 together resulted in 10- to 20-fold the β-galactosidase activity seen in the presence of Ras1 alone. When the $D\text{-}Raf^{C110}$ mutation is introduced, β-galactosidase activity is reduced to that associated with Ras1 alone. The D-Raf CR1 domain alone in this assay did not cause measurable activation.

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