The \textit{Drosophila} 14-3-3 protein Leonardo enhances Torso signaling through D-Raf in a Ras1-dependent manner

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SUMMARY

14-3-3 proteins have been shown to interact with Raf-1 and cause its activation when overexpressed. However, their precise role in Raf-1 activation is still enigmatic, as they are ubiquitously present in cells and found to associate with Raf-1 in vivo regardless of its activation state. We have analyzed the function of the \textit{Drosophila} 14-3-3 gene \textit{leonardo} (\textit{leo}) in the Torso (\textit{Tor}) receptor tyrosine kinase (RTK) pathway. In the syncytial blastoderm embryo, activation of Tor triggers the Ras/Raf/MEK pathway that controls the transcription of \textit{tailless} (\textit{tll}). We find that, in the absence of Tor, overexpression of \textit{leo} is sufficient to activate \textit{tll} expression. The effect of \textit{leo} requires D-Raf and Ras1 activities but not KSR or DOS, two recently identified essential components of \textit{Drosophila} RTK signaling pathways. Tor signaling is impaired in embryos derived from females lacking maternal expression of \textit{leo}. We propose that binding to 14-3-3 by Raf is necessary but not sufficient for the activation of Raf and that overexpressed \textit{Drosophila} 14-3-3 requires Ras1 to activate D-Raf.

INTRODUCTION

The 14-3-3 proteins are a family of small acidic molecules that are highly conserved among diverse species and ubiquitously expressed in many, if not all, tissues (Aitken et al., 1992). These proteins are involved in a variety of biological processes including activation of tryptophan and tyrosine hydroxylases, protein kinase C regulation, Ca\textsuperscript{2+}-dependent exocytosis and cell cycle control (Ford et al., 1994). Recently, they have been suggested to play roles in regulating signal transduction pathways. 14-3-3 proteins have been shown to physically associate with many signaling molecules and the physiological significance of such interactions has been investigated for the Raf-1 kinase (Morrison, 1994; Aitken, 1995). Activation of Raf-1 requires its association with the active GTP-bound form of Ras (Moodie et al., 1993; Egan and Weinberg, 1993). Interaction between the two proteins is not sufficient for activation, however, as mixing GTP-Ras and Raf-1 in vitro does not lead to Raf-1 activation. The observation that membrane-associated forms of Raf-1 are constitutively active led to the proposal that the function of activated Ras is to translocate Raf-1 to the membrane where it becomes activated by an unknown factor (Stokoe et al., 1994; Leever et al., 1994). The finding that 14-3-3 proteins associate with Raf-1 and are able to activate Raf-1 when expressed in yeast and \textit{Xenopus} oocytes, raised the possibility that 14-3-3 is the long sought activator of Raf-1. Co-expression of 14-3-3 and Raf-1 in yeast cells or \textit{Xenopus} oocytes, a 'reconstituted system', has demonstrated that 14-3-3 proteins can increase the kinase activity of mammalian Raf-1 (Freed et al., 1994; Fantl et al., 1994). Conversely, deletion of the yeast 14-3-3 gene BMH1 blocks Ras overexpression-induced activation of Raf-1 in yeast cells (Irie et al., 1994), suggesting that Ras may require 14-3-3 to activate Raf. However, in vitro experiments using purified proteins were unable to show that it is the binding of 14-3-3 to Raf-1 that activates the Raf-1 kinase. Further, in vivo, 14-3-3 proteins appear to constitutively associate with both activated and inactivated forms of Raf-1 (Fu et al., 1994; Li et al., 1995). Therefore the mechanism by which overexpression of 14-3-3 activates Raf-1 in yeast and \textit{Xenopus} oocytes is still not resolved. Further, it remains to be determined whether activation of Raf-1 by overexpressed 14-3-3 proteins requires the participation of additional signaling molecules, such as Ras. Finally, it is not clear whether 14-3-3 is an obligatory component of the biological processes that utilize signal transduction through Raf-1 activation.

D-Raf is the \textit{Drosophila} homologue of the mammalian Raf-1 and contains three highly conserved regions (CR1, CR2 and CR3) that are shared among other Raf family members (Ambrosio et al., 1989b). Mammalian Raf-1 is able to substitute for D-Raf in transducing Tor signals, indicating that Raf-1 and D-Raf can be regulated by the same set of upstream molecules and are able to recognize the same downstream substrates (Casanova et al., 1994; A. Brand and N. Perrimon, unpublished data). Raf-1 contains two 14-3-3 binding motifs that encompass serine residues 259 and 621. A phosphoserine
peptide derived from sequences surrounding serine 621 can disrupt Raf-1/14-3-3 interaction and inhibit Raf-1 function in *Xenopus* oocytes (Muslin et al., 1996). D-Raf has equivalent serine residues at position 388 and 743. Phosphorylation of serine 743 has been shown to be essential for D-Raf function in *Drosophila* embryos (Baek et al., 1996). These data suggest that D-Raf may also directly interact with 14-3-3 proteins in vivo for its normal function.

In *Drosophila*, a 14-3-3 protein encoded by the *leonardo* (*leo*) gene has recently been characterized (Skoulakis and Davis, 1996). We decided to analyze the role of this protein in the Torso (Tor) RTK signaling pathway (also known as the terminal pathway; reviewed in Perrimon and Desplan, 1994; Duffy and Perrimon, 1994; Perrimon, 1994; Dickson and Hafen, 1994). Tor transduces signals through the evolutionarily conserved Ras/Raf/MEK/MAPK module and functions in cell fate specification in the early *Drosophila* embryo. Tor is required for defining the embryonic terminal cell fates. Tor mRNA is maternally provided. A localized ligand activity activates Tor only at the termini (Stevens and Nusslein-Volhard, 1991; Sprenger and Nusslein-Volhard, 1992; Casanova and Struhl, 1993). Different levels of Tor activity specify distinct terminal elements, suggesting that Tor activity is graded and plays an instructive role in establishing the terminal pathway; reviewed in Perrimon and Desplan, 1994; Davis, 1996). We decided to analyze the role of this protein in blocking Raf-1/14-3-3 interaction and inhibit Raf-1 function in *Drosophila* embryos (Baek et al., 1996). These data suggest that D-Raf may also directly interact with 14-3-3 proteins in vivo for its normal function.

In *Drosophila*, a 14-3-3 protein encoded by the *leonardo* (*leo*) gene has recently been characterized (Skoulakis and Davis, 1996). We decided to analyze the role of this protein in the Torso (Tor) RTK signaling pathway (also known as the terminal pathway; reviewed in Perrimon and Desplan, 1994; Duffy and Perrimon, 1994; Perrimon, 1994; Dickson and Hafen, 1994). Tor transduces signals through the evolutionarily conserved Ras/Raf/MEK/MAPK module and functions in cell fate specification in the early *Drosophila* embryo. Tor is required for defining the embryonic terminal cell fates. Tor mRNA is maternally provided. A localized ligand activity activates Tor only at the termini (Stevens and Nusslein-Volhard, 1991; Sprenger and Nusslein-Volhard, 1992; Casanova and Struhl, 1993). Different levels of Tor activity specify distinct terminal elements, suggesting that Tor activity is graded and plays an instructive role in establishing the terminal pattern (Casanova and Struhl, 1989). At the molecular level, graded activity of Tor is reflected by the differential expression domains of *tailless* (*ttl*) and *huckebein* (*hkb*), two Tor target genes (Pignoni et al., 1990, 1992; Weigel et al., 1990). *hkb* is expressed in a smaller domain than *ttl*, which likely reflects differential responses of the *ttl* and *hkb* promoters to the strength of the Tor signals (Perkins and Perrimon, 1991).

The Tor RTK is activated by ligand binding, which triggers dimerization and trans-phosphorylation of the receptor monomers. The phosphotyrosines serve as docking sites for cytoplasmic adaptor proteins such as the *Drosophila* protein DRK/Grb2, which links the cytoplasmic domain of an activated Tor to the guanine releasing factor protein, encoded by the gene *son-of-sevenless* (*sos*; Olivier et al., 1993; Simon et al., 1993; Bonfini et al., 1992). SOS activates Ras1 by exchanging GDP for GTP on Ras1, whereas the GTPase-activating protein, Gap-1, promotes the reverse nucleotide exchange reaction and negatively regulates Ras1 (Gaul et al., 1992). The effect of overexpression of *leo* requires Ras1 to activate the Tor pathway and its ability to enhance Tor signaling is augmented by removing Gap1, a negative regulator of Ras1 (Gaul et al., 1992). The effect of overexpression of *leo* on Tor signaling is not affected by mutations in either *ksr* or *dos*, suggesting that 14-3-3 does not act upstream of these molecules. Finally, by examining embryos derived from germline clones homozygous for a mutant allele of the *leo* gene, we show that *leo* is essential for Tor signaling. Results from this and other studies suggest that 14-3-3 protein functions as a cofactor that requires at least Ras to mediate Raf activation.

**MATERIALS AND METHODS**

**Fly stocks, production of germline clone embryos and heat-shock treatment**

*tor* null mutant embryos were collected from females homozygous for *tor*<sup>NR1</sup>, a protein null allele of *tor* (Sprenger and Nusslein-Volhard, 1992; Sprenger et al., 1993). The ‘FLP-DFS’ technique (Chou and Perrimon, 1992, 1996; Hou et al., 1995) was used to produce embryos derived from germline clones homozygous for mutant alleles of *D-Raf*<sup>1-29</sup> (a protein null allele of *D-Raf*; Sprenger et al., 1993), *Ras1<sup>lacZ40B</sup>* (a protein null allele of *Ras1*; Hou et al., 1995), *ksr<sup>TM6</sup>* and *dos<sup>R31</sup>* (Raabe et al., 1996), *Gap<sup>B2</sup>* (Gaul et al., 1992) and *leo<sup>P1188</sup>* (Skoulakis and Davis, 1996), respectively. *ksr<sup>TM6</sup>* was identified in an ethyl-methanesulfonate screen for zygotic lethal mutations associated with specific maternal effect phenotypes. It behaves genetically as a null mutation (N. Perrimon, unpublished). The molecular nature of the genetically null *ksr* and *dos* allele that we used in this study is not known.

To determine the effect of overexpression of *leo* on embryonic development, the hsp70-*leo* gene was introduced from the father into embryos by crossing females of the appropriate genotypes with males homozygous for the hsp70-*leo* transgene. 0-1 hour old embryos were collected on agar plates and allowed to continue development for an additional hour at 25°C. They were heat shocked by floating the plates in a 34°C water bath for 5 minutes, allowed to develop for 20 minutes at 25°C and then fixed, or allowed to develop for 24 hours for cuticle preparation.

To analyze the cuticular phenotypes of embryos lacking maternal *leo* product, germline clones of *leo* mutations were induced using the ‘FLP-DFS’ technique. Two *leo* alleles, *leo<sup>P1188</sup>* or *leo<sup>P1375</sup>* were used in this study (Skoulakis and Davis, 1996). Females that contained *leo*...
germline clones were crossed either to wild-type males or backcrossed to the respective leo heterozygotes. The cuticle phenotypes and the defects revealed by DAPI and tll stainings were not significantly affected by the paternal contribution. Irrespective of the paternal contribution, about 50% of embryos failed to develop cuticles, while the remainder showed variable cuticular defects that included missing and/or fusion of denticle bands. A wild-type copy of leo introduced from the father only affected the hatching rate slightly. When females with leoP1188 germline clones were crossed to leoP1188/+ males, 2.5% of the embryos hatched (n=200). When similar females were crossed to +/- males, 5% hatched (n=200). The leoP1375 allele appeared weaker than leoP1188 in this analysis because the hatching rate was 7.5 and 15%, respectively.

Plasmids and fly transformants
The leo cDNA (Swanson and Ganguly, 1992) was excised as a 780 bp fragment and was inserted into the pCaSpeR-hs vector for P-element-mediated germline transformation. Three independent transformant lines were obtained, one located on the second and two on the third chromosomes, respectively. All transformant lines behave similarly as they cause lethality of early embryos following a 10 minute heat shock at 37°C. They were designated as hsp70-leo1-3.

The experiments were conducted using line #3.

Rp-leoNRE was constructed as follows. The 780 bp leo cDNA fragment was ligated to a 150 bp hNRE fragment (Wharton and Struhl, 1991) to generate leoNRE(+). (+) designates the orientation of the NRE fragment. A 1.4 kb RNA polymerase promoter fragment (Harrison et al., 1992) was ligated to leoNRE(+) and pUAST (Brand and Perrimon, 1993) to create Rp-leoNRE(+), which was used for P-element mediated germline transformation. A third chromosome-located transgene, Rp-leoNRE(-5), was used in the following experiment. FLP(12); FRT; lelp1188/FRT; ovgP1; Rp-leoNRE(-5)/+ female flies were constructed to analyze the effect of Rp-leoNRE on embryonic development.

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 Tautz and Pfeifle (1989). For Fkh antibody staining, a Guinea pig antibody (gift from P. Carrera) was used at 1:100 dilution and was visualized by using the Vectastain ABC kit. Embryos were mounted in either Euparal (Carolina Biological Supply), 70% glycerol (for DAPI staining) or Hoyer’s mount (for cuticle preparations) and were photographed with a Zeiss Axiophot microscope with Nomarski, fluorescence or dark-field optics, respectively.

RESULTS

Overexpression of leo expands tll expression in wild-type and tor embryos, but not in D-Raf null embryos
We used tll expression to monitor the Tor signaling pathway and to evaluate the effect of overexpression of 14-3-3 proteins on the activation of D-Raf. We induced the expression of the leo gene in early Drosophila embryos from a hsp70-driven transgene and examined its effects on tll expression levels by in situ hybridization. In this study, we focus only on posterior tll expression as, in the anterior, tll is also activated by the Bicoid pathway (Pignoni et al., 1992). In wild-type embryos at late syncytial blastoderm stages, the posterior tll expression domain extends from the posterior tip to 15% egg length anteriorly (Fig. 1A; Pignoni et al., 1992; Hou et al., 1995). In embryos derived from wild-type females crossed to males homozygous for the hsp70-leo transgene, we detected an expansion of the tll expression domain in both anterior and posterior regions of the embryo following a brief heat-shock treatment at precellularization stages (Fig. 1B), suggesting that overexpression of leo enhances Tor signaling. Normally, in embryos laid by females homozygous for torXK1, a null allele, little or no tll expression can be detected in the posterior region (Fig. 1C; Sprenger and Nusslein-Volhard, 1992; Sprenger et al., 1993; Pignoni et al., 1992; Lu et al., 1993). However, when these torXK1 embryos contained a paternal copy of the hsp70-leo transgene and were heat shocked prior to blastoderm formation, we were able to detect tll expression in the posterior region of the embryo (Fig. 1D), while little or no expression was seen in the same embryos that were not heat shocked, or those that were without the hsp70-leo transgene treated in parallel (Fig. 1C). The expression of tll was only detected in the terminal embryonic regions, as was previously observed following ubiquitous expression of an activated human Raf-1 in torXK1 embryos (Casanova et al., 1994; A. Brand and N. Perrimon, unpublished). Consistent with the tll expression results, overexpression of leo in wild-type embryos resulted in deletions of denticle bands (Fig. 2B), a cuticular defect that is similar to that associated with weak tor gain-of-function alleles and ectopic tll expression (Klingler et al., 1988; Strecker et al., 1989; Steingrimsson et al., 1991). In addition, we were able to modestly rescue torXK1 cuticles by heat-shock induction of leo (cf. Fig. 2C, D). These results indicate that overexpression of leo enhances but cannot completely restore Tor activity.

To address whether D-Raf activity is required for the effect of overexpressed 14-3-3 proteins, we generated germline clone embryos homozygous for D-Raf1-29 (throughout the text, we refer to such embryos as D-Raf mutant embryos), a null allele of D-Raf (Ambrosio et al., 1989a; Pignoni et al., 1992; Lu et al., 1993; see Materials and Methods), and examined the effects of overexpression of leo on tll expression in these embryos. As in torXK1 embryos, D-Raf null embryos show no tll expression in the posterior domain. In contrast to results obtained from tor null embryos, the hsp70-leo transgene was unable to activate tll expression in D-Raf null embryos (Table 1; picture not shown), suggesting that Leo acts through D-Raf to activate tll transcription. These results indicate that high levels of 14-3-3 proteins are able to activate signal transduction through D-Raf in the absence of a wild type or an activated RTK. Genetically, the leo gene functions downstream of Tor and upstream of D-Raf. Taken together with the previous biochemical evidence that the mammalian 14-3-3 proteins associate with Raf-1, it is likely that the enhancement of tll expression seen in wild-type or tor null embryos caused by overexpression of leo is due to the activation of D-Raf by elevated levels of the Drosophila 14-3-3 proteins encoded by the leo gene.

Expansion of tll expression by overexpression of leo is dependent on Ras1 and is enhanced by removing Gap1, but is independent of KSR and DOS
To investigate the epistatic relationship between 14-3-3 and other molecules involved in Tor signaling, we examined the changes in the levels of tll expression following heat-shock induction of leo in embryos mutant for Ras1, Gap1, ksr and dos, respectively. About 20% of Ras1 null mutant embryos show residual tll expression that extends from the posterior end

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to approximately 5% EL. This residual \( tll \) expression has led to the proposal of the existence of Tor signaling of a \( Ras1 \)-independent pathway that contributes to full D-Raf activation (Hou et al., 1995). When \( Ras1 \) null embryos containing a paternal copy of the hsp70-\( leo \) transgene were heat shocked, we found no significant changes in either the percentage of embryos that show residual \( tll \) expression or the extent of residual \( tll \) expression (Fig. 3A,B; Table 1). These observations suggest that the ability of overexpressed \( leo \) to activate D-Raf requires \( Ras1 \). We propose that the ability of overexpressed \( leo \) to activate D-Raf in the absence of Tor is due to the presence of a small amount of GTP-bound form of activated \( Ras1 \) that is in equilibrium with the majority of GDP-bound \( Ras1 \) (see Discussion). Such a hypothesis predicts that removing the GTPase-activating protein (Gap1), which catalyzes conversion of GTP- to GDP-bound Ras, would shift the equilibrium in favor of GTP-Ras and therefore would enhance 14-3-3-mediated D-Raf activation. Consistent with this model, induction of hsp70-\( leo \) in embryos null for Gap1 caused a broad expansion of \( tll \) expression towards the center of the embryo (Fig. 3C,D). The stripy appearance of \( tll \) expression in Gap1 null embryos following \( leo \) induction may reflect the difference in the responsiveness of the \( tll \) promoter to activation in different regions of the embryo.

Recently, the \( ksr \) and \( dos \) genes have been implicated in Tor signaling, although their respective roles remain poorly understood (see Introduction). We analyzed the requirement for these gene activities in mediating the effect of overexpressed \( leo \). Embryos derived from germline clones homozygous for a \( ksr \) null mutation have severe terminal class defects that are similar to those exhibited by \( tor \) embryos (Therrien et al., 1995). Similarly, germline clones for a loss-of-function allele of \( dos \) give rise to embryos with defects in terminal structures; however, these are weaker than in \( tor \) or D-Raf loss-of-function mutants (Raabe et al., 1996). As in \( Ras1 \) mutant embryos, we detected some residual \( tll \) expression in either \( ksr \) or \( dos \) mutant embryos (Fig. 3E,G). However, unlike \( Ras1 \) null embryos, heat-shock induction of the hsp70-\( leo \) transgene in either \( ksr \) or, to a lesser extent, \( dos \) mutant embryos caused expansion of the \( tll \) expression domain (Fig. 3F,H; Table 1). These results suggest that overexpressed \( leo \) does not require either KSR or DOS to activate D-Raf.

**leo activity is essential for Tor signaling**

Animals homozygous for \( leo \) mutations and derived from heterozygous mothers die before hatching as morphologically normal embryos (Skoulakis and Davis, 1996). In order to investigate whether \( leo \) is required for Tor signaling, we analyzed the phenotype of embryos derived from female germline clones homozygous for \( leo^{P1188} \), a loss-of-function allele of \( leo \) (see Materials and Methods). These embryos, referred to as \( leo \) mutant embryos, showed variable phenotypes...
that are not significantly different whether or not the embryos contained a wild-type copy of the leo gene from the father (Table 2 and Materials and Methods). Approximately half of the unhatched embryos did not develop cuticles, the remaining developed cuticles with various segmentation defects that include missing and/or fused denticle bands (Fig. 4B). In these embryos, the Filzkörper, a marker of posterior terminal cell fates, appeared wild type (see Perkins and Perrimon, 1991; Fig. 4A,B). DAPI staining showed that about 50% of leo mutant embryos appeared to stop development during syncytial blastoderm stages and that they contained many fewer and occasionally fused nuclei probably reflecting a function of leo during early nuclei divisions. These embryos failed to cellularize (Fig. 4C,D) and probably do not develop cuticles. When examined for tll expression, the leo P1188 mutant embryos that had stopped development during syncytial blastoderm stages showed no tll expression in either anterior or posterior regions. Those that reached the late syncytial and cellular blastoderm stages fell into two classes (Table 2): 60% exhibited wild-type levels of tll expression while the remainder showed a reduction in posterior tll expression (Fig. 4E,F). 40% of leo P1188 mutant embryos showed posterior tll expression at about 11% of egg length (compared to

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<th>Table 1. Effects of leo overexpression on tll expression in mutant embryos</th>
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<td><strong>tll expression levels</strong></td>
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<td>Genotypes</td>
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<td>ksrTM6</td>
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<td>dosR31</td>
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The number of embryos analyzed for tll expression are shown. As a positive control for staining, only embryos that showed detectable tll staining anteriorly were scored. Anteriorly, tll is controlled by both Bicoid and the Tor pathway. In a tor null mutant, tll expression is expanded anteriorly and not expressed posteriorly (Pignoni et al., 1992).

tll expression levels were categorized as followed: −, no more than 3 cells in the posterior expressing tll; +, more than 3 cells but less than 5% of the egg length from the posterior tip; ++, more than 5% of egg length expressing tll in the posterior region.

Genotypes refer to that of the female germline cells from which the embryos were derived.

leo overexpression was induced from a hsp70-leo transgene by heat shocking embryos at 34°C for 5 minutes.

Control embryos either had identical genotypes to the experimental ones but were not heat shocked, or they did not contain the hsp70-leo transgene but endured the same heat-shock treatment. Results from the above two types were indistinguishable and were combined.

15% in wild type; see Fig. 4E,F). In conclusion, the phenotypic variability of leo P1188 mutant embryos suggests two distinct roles for Leo during embryogenesis. First, embryos that have received the least amount of maternal leo gene product fail to cellularize properly, implying a role for Leo in cell division. Second, examination of tll expression in embryos that form correct blastoderm allows us to detect a positive role for Leo in Tor signal transduction. The variability in the phenotype of leo mutant embryos could reflect that either leo P1188 is not a

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<th>Table 2. Phenotypic classes of leo mutant embryos and rescue of the leo maternal effect phenotype by Rp-leoNRE</th>
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<tr>
<td><strong>leo</strong> P1188 GLC</td>
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<td>Cuticle preparation</td>
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<td>Segmentation defects</td>
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<td>Wildtype looking</td>
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<td>Missing posterior and/or shortened Filzkörper</td>
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<td>Embryos scored</td>
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<td>DAPI staining</td>
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Percentages of indicated phenotypic classes are shown (see Materials and Methods).

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complete null or that leo is functionally redundant with another.

To determine whether the reduction in till expression seen in some of the leoP1188 mutant embryos has any effects on the development of internal posterior structures, we examined the development of the posterior midgut, hindgut and Malpighian tubules using an antibody against the Forkhead (Fkh) protein (Weigel et al., 1989). These structures are derived from the posterior-most region of the embryo and are the first ones to be affected by a reduction in Tor signaling (see Perkins and Perrimon, 1991; Diaz et al., 1996 for details on the fate map of posterior structures with respect to till expression). We found that 50% (n=26) of leoP1188 mutant embryos were missing the posterior midgut, hindgut and Malpighian tubules (Fig. 4G,H), which is reminiscent of the defects detected in Draf or till hypomorphic mutants as well as csw null mutants (Perkins et al., 1992, 1996; Melnick et al., 1993). These results indicate that a subset of posterior structures, dependent on Tor signaling, are missing in about half of the leo mutant embryos that develop beyond the blastoderm stage.

In order to rescue the cellularization defect in leo null embryos, we expressed uniformly a leo gene that contained a Nanos-response element (NRE) to restrict translation of Leo selectively to the anterior region of early embryos. The NRE that we used was identified in the 3¢ untranslated regions of the maternal hunchback (hb) and bicoid mRNAs and is responsible for Nanos-mediated translational inhibition and degradation of hb mRNA in the posterior region of early embryos (Wharton and Struhl, 1991). This NRE has been shown to be sufficient for mRNA degradation when fused to a heterologous mRNA (Wharton and Struhl, 1991). Females that carry both leoP1188 germline clones and a maternally expressed leo transgene containing an NRE at the 3¢ end were generated (RpleoNRE, see Materials and Methods). 18% of the embryos derived from such females showed a wild-type anterior region but were missing part or all of the posterior region (Fig. 5B; Table 2), and 56% of these ‘anteriorly rescued’ embryos showed shortened Filzkörper (Fig. 5B; compare with Fig. 4A). The shortened Filzkörper phenotype is consistent with the in situ hybridization results that showed that a fraction of embryos derived from leo germline clones had reduced posterior till expression domains (see Perkins and Perrimon, 1991). Without the rescue with Rp-leoNRE, these embryos probably would not develop cuticles, thus we were unable to see the cuticular consequences of the reduction in posterior till expression. 20% of these anteriorly rescued embryos were completely devoid of posterior till expression, while till was expressed anteriorly (Fig. 4A; Table 2). Interestingly, the level of leo expression from the transgene was undetectable by RNA in situ using a probe specific to the 3¢ UTR of the transgene. We conclude that a low-level of rescuing leo activity in the anterior allowed us to visualize a tor null phenotype in terms of till expression at
the posterior and are consistent with the proposal that leo is an integral component of the Tor pathway.

**DISCUSSION**

Much of our knowledge of the function of 14-3-3 proteins in Raf activation comes from studies using yeast and *Xenopus* oocytes. To evaluate the functions of 14-3-3 proteins in an in vivo biological process, we have analyzed the role of the *Drosophila* 14-3-3 gene, leo, in Tor signaling. The Tor pathway allows a quantitative assessment of the disturbances to D-Raf activation (Hou et al., 1995). *tll* expression in the posterior region of the embryo is solely dependent on Tor signaling. Loss of *tor* or *D-Raf* gene activities eliminates *tll* expression in this region, while gain-of-function *tor* mutations cause an expansion of the posterior *tll* domain towards the middle of the embryo (Ambrosio et al., 1989a; Pignoni et al., 1992; Lu et al., 1993; Casanova and Struhl, 1989; Steingrimsson et al., 1991). We have shown that overexpression of *leo* enhances Ras1-dependent activation of D-Raf and loss of maternal *leo* expression reduces Tor signaling. We also demonstrate that high levels of Leo can activate D-Raf in the absence of KSR or DOS activities. Our results indicate that 14-3-3 proteins are required in the process of Raf activation that requires Ras1.

**14-3-3 and D-Raf activation**

We have inferred that the effects of *leo* overexpression in the Tor pathway are mediated by the activation of D-Raf because the ability of overexpressed *leo* to expand the domain of *tll* expression is suppressed by removing D-Raf gene activity. Since overexpression of *leo* is sufficient to activate *tll* in the absence of Tor, Leo apparently can activate D-Raf in the absence of activated upstream signaling molecules, such as activated Ras1. However, Leo is unable to activate D-Raf in the complete absence of Ras1. Because 14-3-3 proteins have no affinity for Ras and Raf-1 binds only to activated Ras1 (Moodie et al., 1993), it is reasonable to speculate that the activation of D-Raf by Leo in the absence of Tor reflects the presence of a small amount of activated Ras1-GTP that is in equilibrium with the predominant form, Ras1-GDP. In the early embryo, in the absence of Tor activation, a small amount of activated Ras1 may exist that is usually insufficient for D-Raf activation. However, when the level of 14-3-3 protein is increased above normal, binding of 14-3-3 to D-Raf may facilitate association of D-Raf with the endogenous small amount of activated Ras1, and thus lead to D-Raf activation. The presence of a limited quantity of activated Ras1 in the embryo in the absence of activated Tor may explain why, in *tor* null embryos (but not D-Raf mutants), *tll* is occasionally expressed in a few cells at the posterior pole (see Pignoni et al., 1992; this study). This region of the embryo is where *tll* is most sensitive to Tor signaling (Casanova et al., 1994; Hou et al., 1995; this study). This model is also consistent with the effect of overexpressed 14-3-3 in Gap1 mutant embryos, where *tll* expression domains are expanded toward the center of the embryos.

The current model of Raf activation is that activated Ras binds to the CR-1 domain of Raf. As Ras is anchored to the membrane, this binding leads to subcellular relocalization of Raf, which subsequently becomes activated by an unknown mechanism. In light of our results, Leo could be involved in the following steps in D-Raf activation. In one model, it could act to stabilize D-Raf/Ras1 binding, therefore enhancing the association of D-Raf with activated Ras1. In the second model, Leo may be required for the activation of D-Raf by an unknown factor (‘X’) once D-Raf is bound to Ras1. See Discussion for further details.

**Role of 14-3-3 in Drosophila**

**Fig. 6.** Model of D-Raf activation. 14-3-3 constitutively associates with and may provide a structural role to stabilize a certain conformation of D-Raf. Such a conformation may be important for the association of D-Raf/14-3-3 complex with activated Ras1. Alternatively, binding of 14-3-3 to D-Raf may be essential for the activation of D-Raf by an unknown factor (‘X’) once D-Raf is bound to Ras1. See Discussion for further details.
does not absolutely require KSR. Similarly, our studies with DOS, a substrate of the Csw protein tyrosine phosphatase suggest that high levels of 14-3-3 proteins do not require DOS to activate Raf. This result is consistent with the model that Csw and DOS are involved in a pathway that leads to D-Raf activation independently of the Ras-I pathway (see Raabe et al., 1996). However, it is also consistent with the model that Csw and Dos act upstream of Ras-I (see also Lu et al., 1993) since the effects of ectopic leo in dos and tor mutants are similar.

Leo is required in Tor signaling
Several lines of evidence suggest that 14-3-3 proteins are able to facilitate Raf activation. None, however, have unambiguously shown that Raf activation absolutely requires 14-3-3. By reducing the maternal levels of leo gene activity, we have detected a partial reduction in Tor signaling as evidenced by a reduction in the posterior toll expression domain (Fig. 4F). This result indicates that 14-3-3 proteins are normally required in wild-type animals for Tor signaling. By restricting leo expression to the anterior domain, we were able to demonstrate that 14-3-3 proteins are necessary for D-Raf activation (Fig. 5). Our results are consistent with two recent reports that demonstrate a positive requirement for 14-3-3 proteins in the Ras/Raf/MAPK signaling during Drosophila eye development (Chang and Rubin, 1997; Kockel et al., 1997).

14-3-3 proteins are part of a multigene family (Aitken et al., 1992) and, in Drosophila, at least one other 14-3-3 gene, D-14-3-3e, has been recently isolated. Both leo and D-14-3-3e are involved in mediating Ras/Raf signaling in the Drosophila eye (Chang and Rubin, 1997; Kockel et al., 1997). Thus, it is very likely that these two 14-3-3 proteins have redundant functions. This redundancy may explain the variability in the severity of the cellularization and terminal defects phenotypes that we observed. It would be informative to examine the phenotype of embryos doubly mutants for both leo and D-14-3-3e to precisely define the function of these proteins in cellular processes. However, based on observations that leo plays a role in early cell divisions, it is likely that embryos that completely lack 14-3-3 proteins will develop severe mitotic defects and arrest prior to blastoderm formation.

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