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The *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 *Drosophila 14-3-3* gene *leonardo* (*leo*) in the Torso (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 *tailless* (*tll*). We find that, in the absence of Tor, overexpression of *leo* is sufficient to activate *tll* expression. The effect of *leo* requires D-Raf and Ras1 activities but not KSR or DOS, two recently identified essential components of *Drosophila* RTK signaling pathways. Tor signaling is impaired in embryos derived from females lacking maternal expression of *leo*. We propose that binding to 14-3-3 by Raf is necessary but not sufficient for the activation of Raf and that overexpressed *Drosophila* 14-3-3 requires Ras1 to activate D-Raf.

Key words: *Drosophila*, 14-3-3, Ras, Raf, receptor tyrosine kinase

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^{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; Leevers 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 *Xenopus* 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 *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 *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 *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 pattern (Casanova and Struhl, 1989). At the molecular level, graded activity of Tor is reflected by the differential expression domains of *tailless* (*tll*) and *huckebein* (*hkb*), two Tor target genes (Pignoni et al., 1990, 1992; Weigel et al., 1990). *hkb* is expressed in a smaller domain than *tll*, which likely reflects differential responses of the *tll* 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 *Drosophila* Raf-1 homolog, D-Raf, is an effector of Ras1. In mammalian cells, the activated (GTP-bound) form of Ras directly associates with Raf-1 (Vojtek et al., 1993; Moodie et al., 1993). Such an interaction, however, does not result in Raf-1 activation, but rather targets Raf-1 to the plasma membrane, where it is activated by a yet unknown mechanism (Stokoe et al., 1994; Leevers et al., 1994). Once activated, Raf-1 propagates signals through a phosphorylation cascade that involves MAPKK kinase (also called MEK) and MAPK (Moodie and Wolfman, 1994).

Ras1 is probably not the sole activator of D-Raf as, in embryos null for the *Ras1* gene, residual *tll* expression can still be detected in the posterior domain (Hou et al., 1995). Recently, additional components have been found to be involved in D-Raf activation. These include Kinase Suppressor of Ras (KSR; Therrien et al., 1995) and Daughter of Sevenless (DOS; Herbst et al., 1996; Raabe et al., 1996). *ksr* was isolated because mutations in this gene suppress an activated Ras1 but not activated D-Raf. Initially thought to act between Ras1 and D-Raf, KSR was recently found to modulate the signal propagation between Raf, MEK and MAPK via a novel mechanism (Therrien et al., 1996). DOS is a substrate of Corkscrew (Csw; Perkins et al., 1992), and is essential for mediating signal transduction by several *Drosophila* RTKs (Herbst et al., 1996; Raabe et al., 1996). Csw has been shown to directly associate with the Tor cytoplasmic domain independent of DRK binding (Cleghon et al., 1996) and may represent a Ras1-independent pathway for D-Raf activation (Raabe et al., 1996).

We have used the Tor pathway to assess the role of the 14-3-3 protein Leo in D-Raf activation. Here we show that high levels of Leo proteins are able to enhance Tor signaling and are sufficient to activate, to a low level, the terminal pathway in the absence of Tor. This effect probably operates through the activation of D-Raf because terminal pathway activation is blocked by removing D-Raf activity from the embryo. Furthermore, we demonstrate that 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^{XR1}, 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 $D\text{-}Raf^{11\text{-}29}$ (a protein null allele of *D-Raf*; Sprenger et al., 1993), $Ras1^{\Delta C40B}$ (a protein null allele of *Ras1;* Hou et al., 1995), ksr^{7M6}, dos^{R31} (Raabe et al., 1996), $Gap1^{B2}$ (Gaul et al., 1992) and leo^{P1188} (Skoulakis and Davis, 1996), respectively. ksr^{7M6} 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^{P1188} or leo^{P1375} were used in this study (Skoulakis and Davis, 1996). Females that contained *leo*

germline clones were crossed either to wild-type males or back-crossed 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 leo^{P1188} germline clones were crossed to leo^{P1188}/+ males, 2.5% of the embryos hatched (n=200). When similar females were crossed to +/+ males, 5% hatched (n=200). The leo^{P1375} allele appeared weaker than leo^{P1188} 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-*leo*1-3. 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 hbNRE fragment (Wharton and Struhl, 1991) to generate *leo*NRE(+) ((+) designates the orientation of the NRE fragment). A 1.4 kb RNA polymerase promoter fragment (Harrison et al., 1992) was ligated to *leo*NRE(+) and pUAST (Brand and Perrimon, 1993) to create Rp-*leo*NRE(+), which was used for P-element mediated germline transformation. A third chromosome-located transgene, Rp-*leo*NRE-(5), was used in the following experiment. FLP^{12}/+; $FRT^{2R\text{-}G13}$ leo^{P1188}/$FRT^{2R\text{-}G13}$ ovo^{D1}; *Rp-leoNRE-(5)*/+ females were constructed to analyze the effect of *Rp-leo*NRE 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 tor^{XR1}, 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 tor^{XR1} 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 tor^{XR1} 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 tor^{XR1} 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 $D\text{-}Raf^{11\text{-}29}$ (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 tor^{XR1} 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

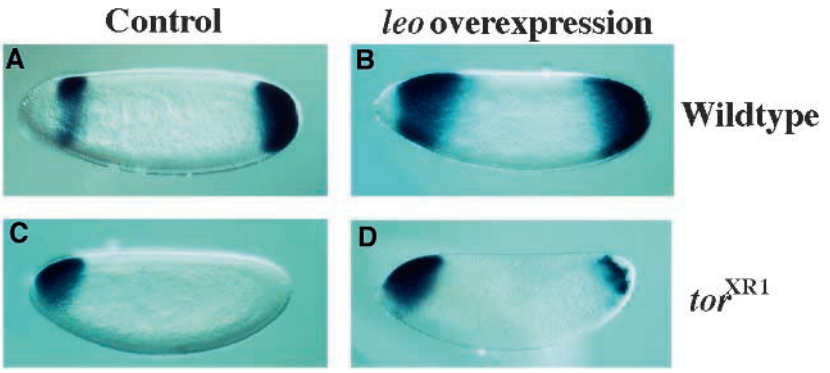

Fig. 1. Changes in *tll* expression levels following heat-shock induction of the hsp70-*leo* transgene. The effects of heat-shock induction of the hsp70-*leo* transgene (B,D) in wild-type (A,B) or tor^{XR1} (C,D) embryos are shown. In wild-type embryos that contain a hsp70-*leo* transgene, *tll* expression is expanded in both the anterior and posterior regions following heat shock (B, *n*=33/102 embryos). In control embryos, which represent either embryos that were not heat shocked (*n*=121 embryos) or were heat shocked in the absence of the hsp70-*leo* transgene (*n*=98), *tll* is not expanded (A). (A) Ventrolateral view. In the case of tor^{XR1} embryos (C,D), 20% showed *tll* expression in the posterior region similar to those shown in D after heat-shock induction of hsp70-*leo* (Table 1). This was not observed in control embryos (C; Table 1). Posterior *tll* expression was not detected in heat shocked embryos derived from homozygous $D\text{-}Raf^{11\text{-}29}$ germline clones that contained the hsp70-*leo*-gene (Table 1). *tll* expression in these embryos was as in C. Only embryos at the late syncytial blastoderm stage that showed positive *tll* at the anterior end (Table 1) were scored.

to approximately 5% EL. This residual *tll* expression has led to the proposal of the existence in 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

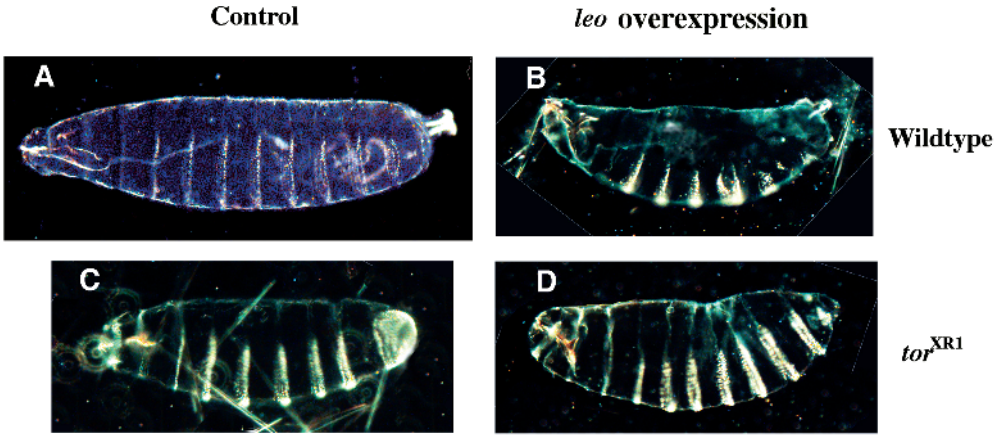

Fig. 2. Effects of *leo* overexpression on larval cuticles. Cuticle preparations of wild-type embryos without (A) or with (B) heat-shock induction (10 minutes at 37°C) of *leo* expression from the hsp70-*leo* transgene. Note the disruption of denticle bands but fairly normal terminal structures in B. Cuticle preparations of embryos from tor^{XR1} homozygous mothers without (C) or with (D) heat-shock induction of *leo* expression. Note the presence of residual A8 and extra posterior structures in D. All control embryos had either 6 or 7 abdominal segments (C).

Table 1. Effects of *leo* overexpression on *tll* expression in mutant embryos

	tll expression levels					
	Control			*leo* overexpression		
Genotypes	–	+	++	–	+	++
tor^{XR1}	119	0	0	115	29	0
D-Raf$^{11-29}$	23	0	0	69	0	0
Ras1$^{\Delta C40B}$	57	18	0	62	19	0
ksr^{7M6}	37	20	0	25	32	10
dos^{R31}	26	22	0	15	13	5

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.

Table 2. Phenotypic classes of *leo* mutant embryos and rescue of the *leo* maternal effect phenotype by *Rp-leoNRE*

	leo^{P1188} GLC	*leo*^{P1188} GLC +*Rp-leoNRE*
Cuticle preparation		
No cuticles	50%	31%
Segmentation defects	45%	37%
Wildtype looking	5%	14%
Missing posterior and/or shortened Filzkörper	0	18%
Embryos scored	400	200
DAPI staining		
Abnormal mitosis and/or nuclear distribution	51%	48%
Wildtype looking	49%	52%
Embryos scored	128	88
tll expression		
No staining	50%	31%
Reduced (to 11% EL)	20%	19%
Wildtype	30%	30%
Missing posterior staining only	0	20%
Embryos scored	182	98

Percentages of indicated phenotypic classes are shown (see Materials and Methods).

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 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 blastoderms allow 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

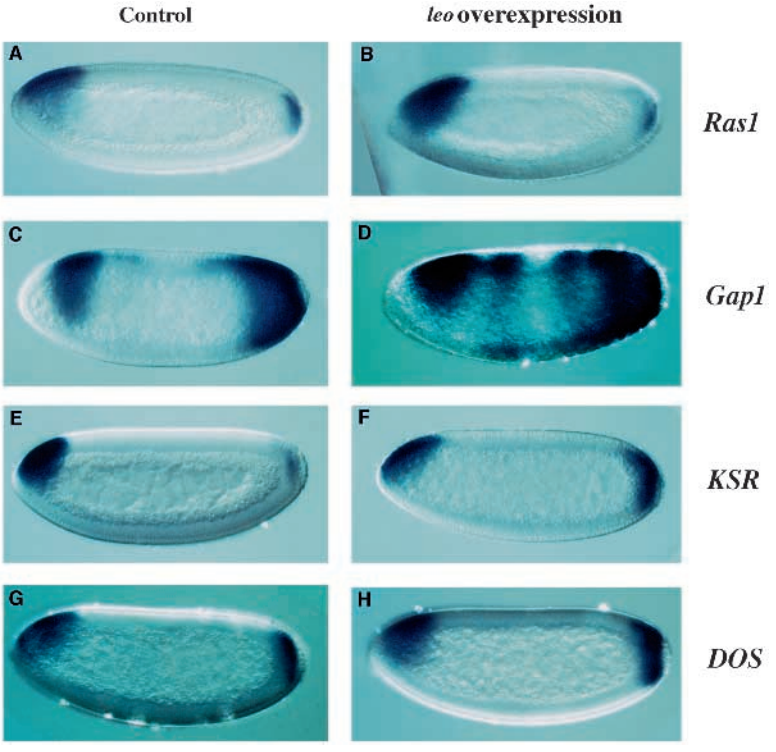

Fig. 3. Effects of heat-shock induction of hsp70-*leo* on *tll* expression in *Ras1*, *Gap1*, *ksr*, or *dos* mutant embryos. *tll* expression in *Ras1* (A,B), *Gap1* (C,D), *ksr* (E,F), and *dos* (G,H) mutant embryos carrying hsp70-*leo* in the absence (A,C,E,G) or following (B,D,F,H) heat shock. Note the increase in *tll* expression in *Gap1* mutant embryos following hsp70-*leo* induction, 38% (n=26) of the embryos showed such a severe expansion.

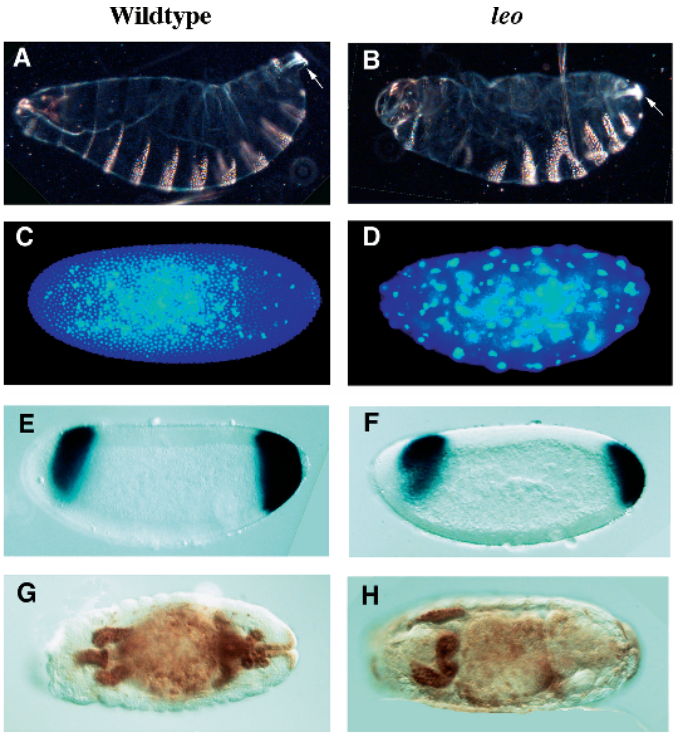

Fig. 4. Phenotypes of *leo*^{P1188} mutant embryos. Cuticle preparations of wild-type embryos (A); embryos derived from homozygous *leo*^{P1188} germline clones (B). Note that the Filzkörper, indicated by arrow, appears wild type in B. DAPI staining of wild-type (C) and *leo*^{P1188} germline clone derived (D) embryos are shown. Note the clumping of nuclei on the surface of the embryo shown in D. (F) In 40% (n=96) of *leo*^{P1188} mutant embryos derived from germline clones, the posterior *tll* expression domain encompasses about 11% of the egg length. Interestingly, most of the these embryos are missing pole cells. In wild-type embryos, *tll* occupies 15% of the egg length (E). (G,H) Internal structures as revealed by anti-Fkh staining. Note the absence of posterior internal structures in *leo* mutant embryos (H) while the anterior internal structures appear normal.

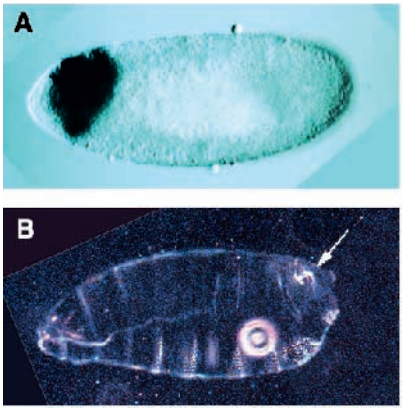

Fig. 5. Restricted expression of *leo* in anterior regions of *leo* mutant embryos. *tll* expression (A) and cuticle phenotype (B) of embryos derived from homozygous *leo*^{P1188} germline clones, which are partially rescued by the Rp-*leo*NRE transgene. Note the presence of *tll* expression in the anterior but not the posterior region of the embryo in A. These embryos apparently have defects in cellularization and probably would not develop any further. The phenotype in B was dependent on the presence of both the *leo*-NRE transgene and maternal homozygosity of *leo*^{P1188,} as it was not observed with either alone. Note that the Filzkörper, indicated by arrow, is greatly reduced in B. In addition, in the presence of Rp-*leo*NRE there was a 40% decrease in the class of *leo*^{P1188} germline clone embryos showing no cuticles and an increase in the hatching rate from 5% to 14%.

complete null or that *leo* is functionally redundant with another *14-3-3* gene (see Discussion).

To determine whether the reduction in *tll* expression seen in some of the *leo*^{P1188} 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 posteriormost 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 *tll* expression). We found that 50% (n=26) of *leo*^{P1188} mutant embryos were missing the posterior midgut, hindgut and Malpighian tubules (Fig. 4G,H), which is reminiscent of the defects detected in *D-raf* or *tll* 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 *leo*^{P1188} germline clones and a maternally expressed *leo* transgene containing an NRE at the 3′ end were generated (*Rp-leo*NRE, 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 *tll* expression domains (see Perkins and Perrimon, 1991). Without the rescue with *Rp-leo*NRE, these embryos probably would not develop cuticles, thus we were unable to see the cuticular consequences of the reduction in posterior *tll* expression. 20% of these anteriorly rescued embryos were completely devoid of posterior *tll* expression, while *tll* was expressed anteriorly (Fig. 4A; Table 2). Presumably these embryos would not have expressed *tll* in the anterior without the rescuing transgene. 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 *tll* 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 Ras (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

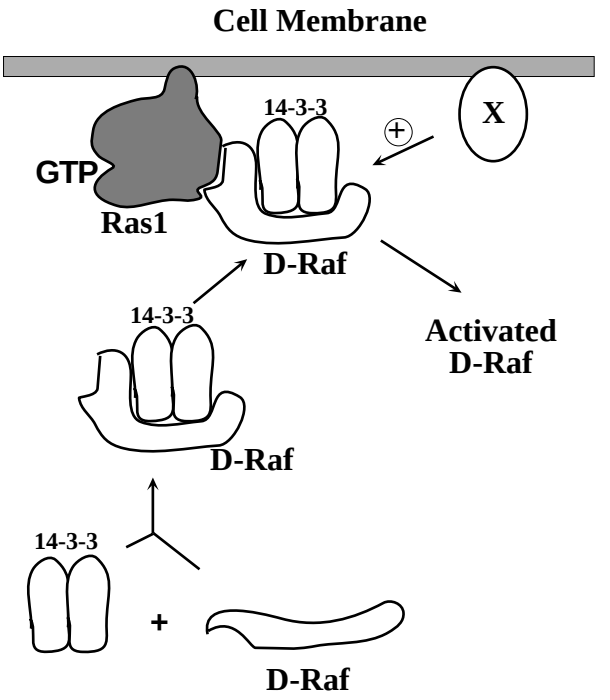

Fig. 6. Model of D-Raf activation. 14-3-3 constitutively associates with and may provide a structural role to stabilize a certain conformation of D-Raf. Such a conformation may be important for the association of D-Raf/14-3-3 complex with activated Ras1. Alternatively, binding of 14-3-3 to D-Raf may 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.

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 activity at the plasma membrane (Fig. 6). These models are consistent with results from coexpression of Raf-1 and 14-3-3 in yeast. When mammalian Raf-1 is overexpressed in yeast cells (yeast does not have a Raf-1 homolog), its activation is limited by the amount of endogenous 14-3-3 proteins (Irie et al., 1994).

It has been suggested that dimerization or oligomerization of Raf-1 is sufficient for its activation and that 14-3-3 proteins contribute to the oligomerization of Raf-1 (Farrar et al., 1996; Luo et al., 1996; Xiao et al., 1995; Liu et al., 1995). According to this model Leo proteins could simply activate D-Raf by causing its oligomerization. We think that this is an unlikely model because our results indicate that the activation of D-Raf following Leo overexpression is dependent on Ras1.

We tested the requirement for KSR and DOS, two novel components of RTK signaling pathways, in mediating the effect of overexpressed *leo*. Genetic studies in *Drosophila* have suggested that *ksr* acts between Ras1 and D-Raf. Additional investigations have proposed that KSR regulates signaling between Raf, MEK and MAPK (Therrien et al., 1995, 1996). Recently, KSR was identified as a ceramide-activated protein (CAP) kinase that directly complexes with Raf-1 and activates Raf-1 by phosphorylation (Zhang et al., 1997). Our results suggests that activation of Raf by overexpression of 14-3-3

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 Ras1 pathway (see Raabe et al., 1996). However, it is also consistent with the model that Csw and Dos act upstream of Ras1 (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 *tll* 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-14-3-3ε*, has been recently isolated. Both *leo* and *D-14-3-3ε* 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-14-3-3ε* 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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