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A Protein Kinase Similar to MAP Kinase Activator Acts Downstream of the Raf Kinase in Drosophila

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Summary

D-raf, a Drosophila homolog of Raf-1, plays key roles in multiple signal transduction pathways. Dsor1, a putative factor downstream of D-raf, was genetically identified by screening of dominant suppressors of *D-raf*. *Dsor1^{Su1}* mapped on X chromosome significantly suppressed the *D-raf* mutant phenotypes, and the loss-of-function mutations of *Dsor1* showed phenotypes similar to those of the *D-raf* null mutations. *Dsor1^{Su1}* also significantly suppressed the mutations of other terminal class genes acting further upstream of *D-raf*. Molecular cloning of *Dsor1* revealed its product with striking similarity to the microtubule-associated protein (MAP) kinase activator and yeast PBS2, STE7, and byr1. Our genetic results demonstrate the connection between raf and the highly conserved protein kinase cascade involving MAP kinase in vivo.

Introduction

Raf-1, encoded by *c-raf*, plays key roles in the transduction of a variety of growth-stimulating transmembrane signals (for reviews, see Li et al., 1991; Rapp, 1991). A Drosophila homolog of the human c-raf-1, *D-raf* has been cloned, and the mutants defective for this gene, *D-raf* (formerly designated as *l(1)raf* or *l(1)pole hole* [*l(1)ph*]), have been identified by a P element–mediated rescue experiment (Nishida et al., 1988; Ambrosio et al., 1989b). The mutant phenotype indicates a major role of *D-raf* in the regulation of cell proliferation as the mammalian *c-raf* (Perrimon et al., 1985; Nishida et al., 1988). *D-raf* is also required for the determination of cell fates at embryonic termini and for development of the compound eye (Perrimon et al., 1985; Nishida et al., 1988). Thus, D-raf acts in multiple signal transduction pathways, among which the terminal system is one of the best systems to elucidate principles of molecular mechanisms of signal transduction (for details see legend to Figure 4).

To understand the roles of D-raf and the significance of phosphorylation reactions in signal transduction, it is crucial to elucidate its substrates. We took a genetic approach to identify new factors downstream of D-raf. We expected that gain-of-function mutations in the genes for D-raf downstream factor would activate pathways without upstream signals so that they would suppress the *D-raf* mutant phenotypes dominantly. We also expected that their loss-of-function phenotypes would at least partially resemble those of *D-raf*. In this article, we report a genetic identification of a gene for a putative factor downstream of the D-raf kinase. Molecular cloning revealed that it encodes a protein kinase similar to the microtubule-associated protein (MAP) kinase activator and suggests a cascade of phosphorylation reactions in the D-raf-mediated signaling pathways.

Results

Genetic Screening for D-raf Downstream Factors

D-raf^{C110} is a weak allele and causes lethality at eclosion or soon after eclosion owing to defects of muscle or nervous system in the thorax (Y. N., unpublished data). *D-raf^{C110}* also showed an eye phenotype: the number of omattidia is reduced, and the eye surface is rough (Perrimon et al., 1985). Flies carrying *D-raf^{C110}* chromosome were treated with ethylmethane sulfonate (EMS), and viable *D-raf^{C110}*/Y males were screened as described in Experimental Procedures. Twenty viable and fertile lines were obtained among about 200,000 flies screened. In this report, we describe one of them.

The eclosion defects and the eye phonotype of *D-raf^{C110}* are fully suppressed in this X-linked mutant. A genetic recombination analysis revealed the presence of the original *D-raf^{C110}* mutation, excluding the possibility of reversion of *D-raf^{C110}*. The downstream of raf suppressor mutation *Dsor1^{Su1}* was located at 27.6 on the genetic map of X chromosome. To exclude further the possibility of duplication of the *D-raf* locus, revertants of *Dsor1^{Su1}* were screened. Disruption of the duplicated locus would cause no serious effect under the genetic background of *D-raf^{+}*, while loss-of-function mutations of a gene for a *D-raf* downstream factor would exhibit phenotypes similar

Table 1. Effects of the *D-raf* and *Dsor1* Mutations on the Rate of Proliferation

Genotype	Number of Twin Spots Analyzed	Mean Number of Cell Divisions		Relative Rate (A/B)	Normalized Rate
		mwh (A)	f^{36a} (B)		
+/M2'	57	6.63 ± 1.47	7.07 ± 1.51	0.946 ± 0.126	1.00
$Dsor1^{Su1}/M2'$	38	6.37 ± 1.39	6.74 ± 1.29	0.944 ± 0.098	1.00
$D\text{-}raf^{1}/M2'$	79	3.41 ± 1.07	5.79 ± 0.99	0.588 ± 0.161	0.62
$D\text{-}raf^{1}\ Dsor1^{Su1}/M2'$	74	5.01 ± 1.28	6.34 ± 1.20	0.793 ± 0.133[a]	0.84
$Dsor1^{r1}/M2'$	13	4.82 ± 1.67	8.28 ± 1.77	0.585 ± 0.166	0.62
$Dsor1^{r2}/M2'$	28	4.80 ± 1.61	8.40 ± 1.30	0.568 ± 0.162	0.60

[a] Suppression is statistically significant at 1% level in t test.

Twin spots formed in wings were analyzed. Cells homozygous for *D-raf* mutations, *Dsor1* mutations, or both were marked with *multiple wing hairs (mwh)*, while cells homozygous for their wild-type allele (control) were marked with $forked^{36a}$ (f^{36a}). The number of cell division was calculated from the number of cells in each clone and the rates of proliferations of the *mwh* (mutant) cells relative to the f^{36a} (control) cells, and their normalized rates are indicated. *M2'* abbreviates the X chomosome: *Dp(1;Y;3)M2'*, $mwh^{+}ve^{+}FR1$, *y w v* f^{36a}.

to those of *D-raf*. Flies double mutant between $D\text{-}raf^{C110}$ and $Dsor1^{Su1}$ were treated with EMS, and two reversions, $Dsor1^{r1}$ and $Dsor1^{r2}$, were obtained. Replacement of $D\text{-}raf^{C110}$ with $D\text{-}raf^{+}$ by genetic recombination revealed lethal mutations in these chromosomes. The lethals failed to complement each other, and detailed genetic mapping located them at the same genetic map position as that of $Dsor1^{Su1}$. Furthermore, their phenotypes are similar to those of null functional *D-raf* mutants as described below.

Effects on Proliferation

One of the major functions of *D-raf* is the regulation of cell proliferation (Perrimon et al., 1985; Nishida et al., 1988). Although the suppressor did not rescue the viability of strong *D-raf* mutants, a significant increase in the size of the imaginal discs was observed (data not shown). To quantitate the suppressing activities, a clonal or twin spot analysis (Postlethwait, 1978; Lawrence et al., 1986) was performed. As shown in Table 1, a 38% reduction of the proliferation rate was observed in $D\text{-}raf^{1}(l(1)raf^{1})$, a null functional allele (Nishida et al., 1988). The proliferation defect of $D\text{-}raf^{1}$ was significantly recovered by $Dsor1^{Su1}$. The interaction of the suppressor with *D-raf* is not allele specific, and the defects in all tested *D-raf* alleles induced by either EMS, methylnitroso urea, or X-ray were similarly rescued (data not shown).

The loss-of-function mutants of *Dsor1* died during larval/pupal stages with severe defects in tissues containing proliferating cells inside the normal-looking larvae as in the *D-raf* null mutants (data not shown). A clonal analysis showed a reduction in rates of proliferation in the cells homozygous for $Dsor1^{r1}$ or $Dsor1^{r2}$ to the proliferation rate of $D\text{-}raf^{1}$ (Table 1). The results indicate that Dsor1, as well as D-raf, is essential for a normal rate of proliferation. A 40% reduction of growth rate would cause considerable phenotypic effects. If we assume that a wing is composed of 30,000 (= $2^{14.9}$) cells, it can be estimated that there are only 478 (= $2^{8.9}$) cells in a mutant wing. This reduced rate of proliferation would explain the observed defects of imaginal discs in the mutant larva. Thus, both D-raf and Dsor1 play major roles in the determination of the rate of proliferation.

Suppression of Embryonic Terminal Defects

A large amount of maternal *D-raf* messenger RNA is stored in the ooplasm, and maternal *D-raf* activity is essential for the determination of cell fates at both anterior and posterior termini of the embryo (Nishida et al., 1988; Ambrosio et al., 1989a). Structures posterior to the seventh abdominal segment are missing in the embryos derived from germline clones homozygous for $D\text{-}raf^{1}$ (Figure 1B). When $Dsor1^{Su1}$ was introduced into the maternal genome, a partial formation of the eighth abdominal segment was observed in 56.7% of embryos (Figure 1C), and even more posterior structures such as filzkörper were formed in 8.7% of embryos (Figure 1D). The suppressor, however, did not affect the anterior defects at all. Zygotic expression of paternally introduced $Dsor1^{Su1}$ did not rescue the terminal defects, suggesting the maternal effect of *Dsor1*.

To examine the role of *Dsor1* in the development of embryonic termini, a germline mosaic analysis was performed. In about half of the embryos derived from germline clones homozygous for either $Dsor1^{r1}$ or $Dsor1^{r2}$, both anterior and posterior structures proved defective (Figure 1E). In the remaining embryos presumably receiving Y chromosome, poor cuticle development was observed (Figure 1F). The results demonstrate the paternally influenced maternal effects of *Dsor1* as observed with *D-raf* (Perrimon et al., 1985; Nishida et al., 1988) and demonstrate that the maternal *Dsor1* is essential for the development of the embryonic termini.

Interactions of *Dsor1* with Terminal Class Genes

If Dsor1 acts downstream of D-raf, it can be expected that $Dsor1^{Su1}$ would also suppress mutations in the terminal class genes encoding the torso (tor) receptor and further upstream factors. Maternal expression of $Dsor1^{Su1}$ suppressed the posterior defects in the embryos mutant for *tor*, *fs(1)pole hole* (*fs(1)ph*), *trunk* (*trk*), or *torso-like* (*tsl*) in a gene dose-dependent manner (Figures 1G–1I). The anterior defects were not rescued in every case. $Dsor1^{Su1}$ did not affect a mutation of *tailless* (*tll*), one of the target genes of the terminal system (data not shown). Revertants of $Dsor1^{Su1}$, $Dsor1^{r1}$ and $Dsor1^{r2}$, lost virtually all their suppressing activity. $Dsor1^{Su1}$ also significantly rescued the

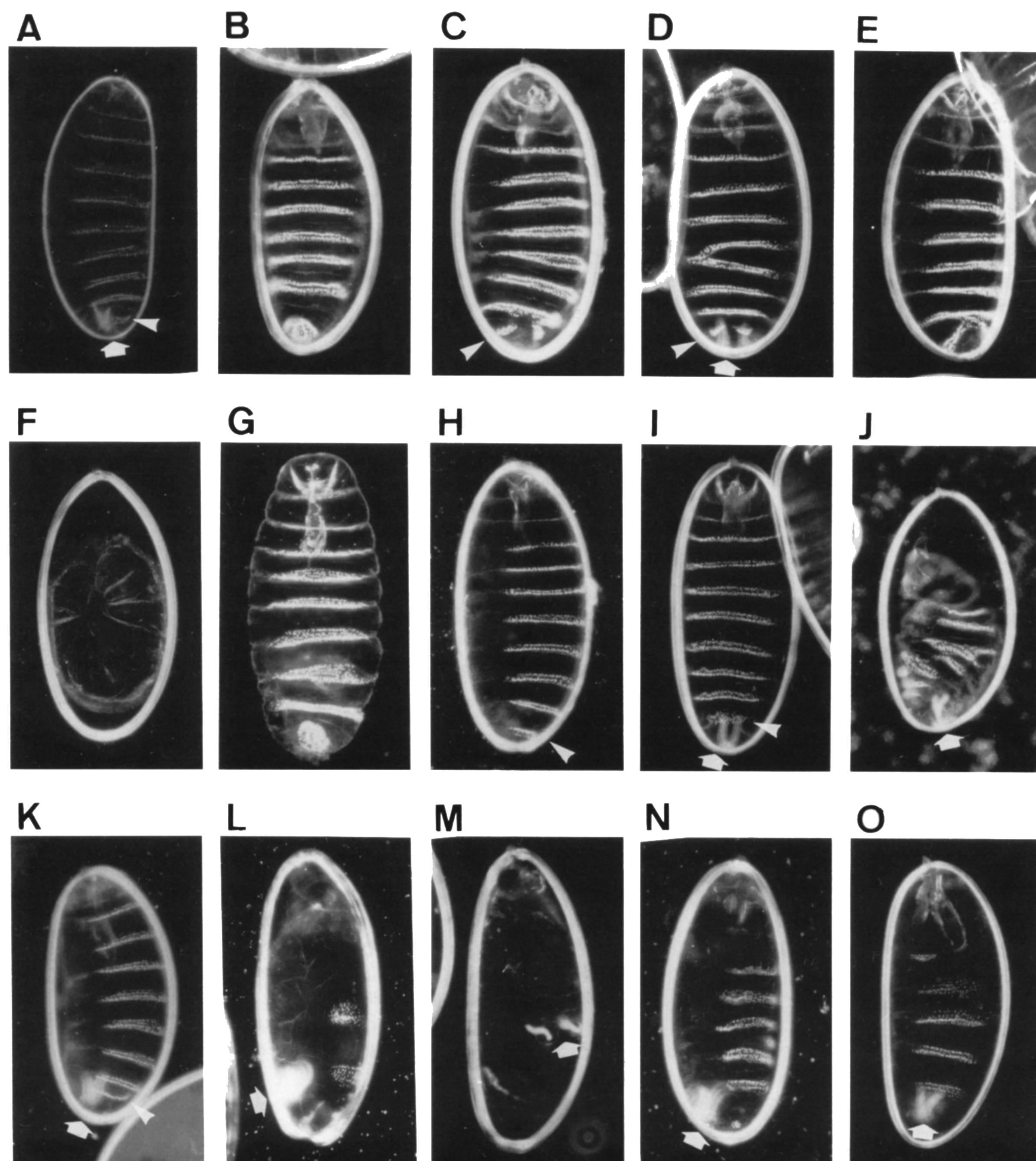


Figure 1. Genetic Interactions of *Dsor1* Mutations with Terminal Class Mutations

The cuticle pattern of a normal embryo is shown in (A). The posterior defect in the embryos derived from germline clones homozygous for $D\text{-}raf^{1}$ (B) was significantly suppressed by maternal expression of $Dsor1^{Su1}$ ([C] and [D]). Half of the embryos derived from germline clones homozygous for $Dsor1^{r1}$ showed terminal defects similar to those of terminal class mutant embryos (E), and poor cuticle development was observed in the remaining embryos (F). The posterior defect in the tor^{PM} (null functional) mutant embryos (G) was significantly suppressed by $Dsor1^{Su1}$, and in about 80% of the embryos laid by $Dsor1^{Su1}/Binsc;tor^{PM}/tor^{PM}$ females, formation of the ventral denticle belt of the eighth abdominal segment (arrowhead) was observed (H). More than 96% of the embryos laid by $Dsor^{Su1}/Dsor1^{Su1};tor^{PM}/tor^{PM}$ females developed the eighth abdominal segment, and in more than 20% of them, more posterior structures such as filzkörper (thick arrow) were also formed (I). Similar dose-dependent suppression by $Dsor1^{Su1}$ was observed with $fs(1)ph^{1901}$, trk^{RA41}, and tsl^{035}. Embryos derived from germline clones homozygous for $E(sev)1A^{e0P}$ showed the "corkscrew" phenotype (J). $Dsor1^{Su1}$ rescued the viability in half of the embryos, and the remaining embryos failed to hatch despite the development of normal cuticle pattern (K). Embryos laid by females homozygous for tor^{RL3} are defective in the segmented central regions, and an average of 1.4 ± 1.3 (N = 276) ventral denticle belts were formed at 20°C (L). $Dsor1^{Su1}$ enhanced the dominant phenotype of tor^{RL3}, and only an average of 0.3 ± 0.6 (N = 138) ventral denticle belts were developed in the embryos laid by $Dsor1^{Su1}/Binsc;tor^{RL3}/tor^{RL3}$ females (M). On the other hand, reduction of the gene dose of $Dsor1^{+}$ or $D\text{-}raf^{+}$ suppressed tor^{RL3}. In the embryos laid by females with genotypes of either $Dsor1^{r2}/Binsc;tor^{RL3}/tor^{RL3}$ (N) or $D\text{-}raf^{1}/Binsc;tor^{RL3}/tor^{RL3}$ (O), averages of 2.0 ± 1.3 (N = 221) or 3.1 ± 1.8 (N = 189) ventral denticle belts were formed, respectively. All embryos were viewed from their ventral or ventrolateral sides and are oriented with anterior ends to the top. Arrowheads and thick arrows indicate the eighth abdominal segments and filzkörpers, respectively. Filzkörpers are out of focus in these pictures.

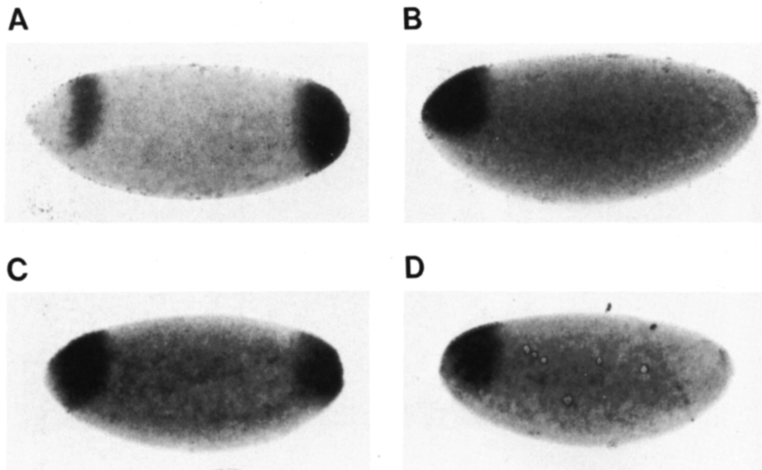


Figure 2. Effects of *Dsor1* Mutations on the Expression Pattern of *tll*

The *tll* transcripts were detected in whole-mount embryos by in situ hybridization. Embryos shown were laid by a Canton S (wild-type) female (A), a $fs(1)ph^{1901}/fs(1)ph^{1901}$ female (B), a $fs(1)ph^{1901}$ $Dsor1^{Su1}/fs(1)ph^{1901}$ $Dsor1^{Su1}$ female (C), and a female with a germline clone homozygous for $Dsor1^{r1}$ (D). Similar *tll* expression patterns as observed in (B) were seen in embryos mutant for tor^{PM} and $D\text{-}raf^{1}$. All embryos are oriented with their anterior ends to the left and dorsal sides up.

defects in the embryos derived from germline clones homozygous for *E(sev)1A*, which is allelic to *corkscrew* (*csw*) encoding a nonreceptor protein-tyrosine phosphatase (Figures 1J and 1K) (Simon et al., 1991; Perkins et al., 1992).

Interactions of the *Dsor1* mutations with a dominant gain-of-function mutation of *tor* were also analyzed. tor^{RL3} is a temperature-sensitive dominant allele and shows phenotypes complementary to those in tor^{PM}: segmented central regions instead of nonsegmented terminal regions are severely affected (Klingler et al., 1988; Strecker et al., 1989; Figure 1L). The dominant tor^{RL3} phenotype was considerably enhanced by $Dsor1^{Su1}$ (Figure 1M), while it was suppressed by reduction of the gene dose of $Dsor1^{+}$ (Figure 1N). A similar dose effect of $D\text{-}raf^{+}$ was also observed (Ambrosio et al., 1989b; Figure 1O). The results suggest that D-raf and Dsor1 function downstream of tor in the expression of the dominant phenotype of tor^{RL3}. To confirm the order of gene action further, a double mutant analysis was performed between tor^{RL3} and $Dsor1^{r2}$. The dominant tor^{RL3} phenotype was abolished, and, instead, there was an observable loss of nonsegmented terminal regions caused by $Dsor1^{r2}$ (data not shown). This indicates that Dsor1 functions downstream of tor if they function in the same pathway.

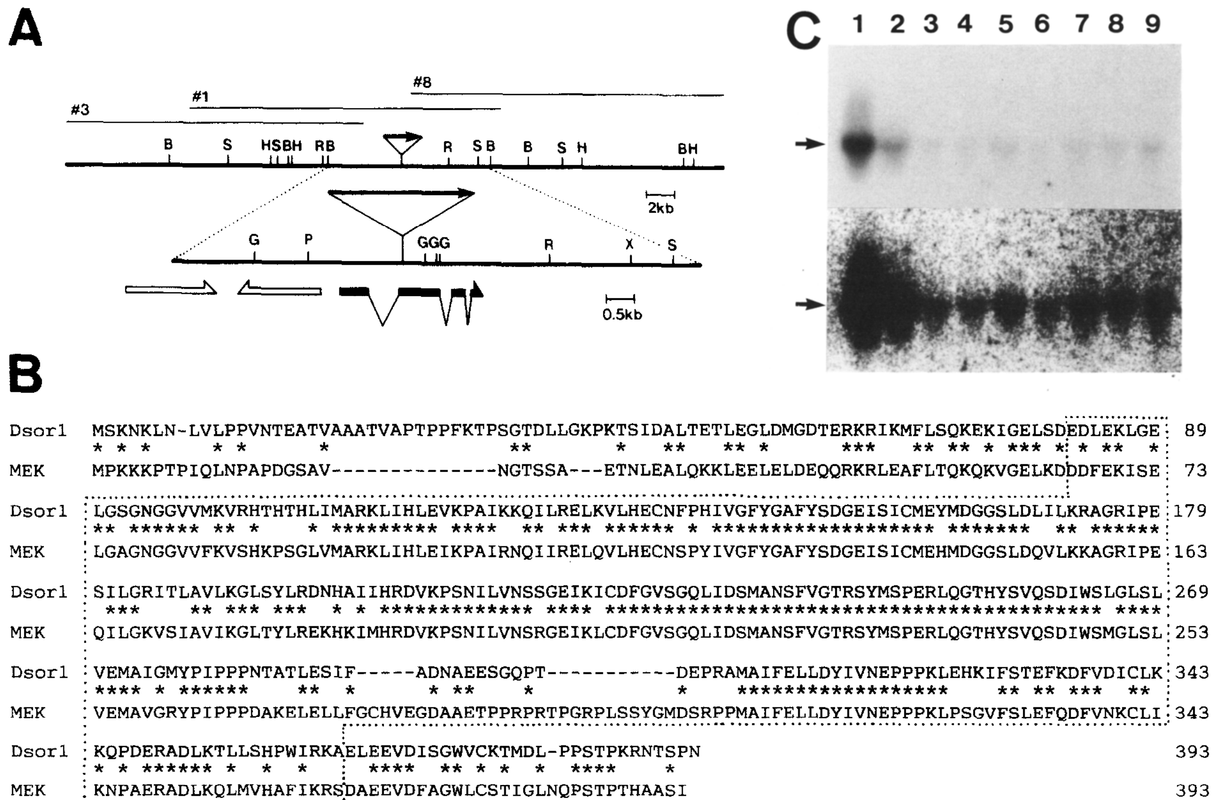
Effects on *tll* Gene Expression

tll is expressed in a pattern in the blastoderm embryo largely consistent with the mutant phenotype: a posterior cap and an anterior–dorsal stripe (Pignoni et al., 1990; Figure 2A). In situ hybridization of whole embryos revealed that the mutations in the terminal class maternal-effect genes, *fs(1)ph*, *tor*, and *D-raf*, affect the *tll* expression pattern similarly: the posterior cap is lost, and the anterior–dorsal stripe is expanded anteriorly (Figure 2B). The result suggests that the maternal products of the terminal class genes regulate the expression of *tll* positively at the posterior terminus and negatively at the anterior domain. The posterior expression pattern of *tll* was restored in the embryo double mutant between $fs(1)ph^{1901}$ and $Dsor1^{Su1}$, while the aberrant anterior expression pattern remained unaffected, consistent with the morphological observations (Figure 2C). The result indicates that suppression of the posterior defects by $Dsor1^{Su1}$ is through its effects on *tll* and strongly suggests that Dsor1 is involved in the regulation of *tll*. In fact, similar aberrant expression patterns of *tll* were observed at both termini in the embryos derived from germline clones homozygous for $Dsor1^{r1}$ or $Dsor1^{r2}$ (Figure 2D). This indicates that Dsor1 is as involved in both positive and negative regulation of *tll* as in other terminal class gene products.

Cloning of the *Dsor1* Gene

To clone the *Dsor1* gene, we have isolated a new mutation, $Dsor1^{Gp158}$, which is noncomplementary to $Dsor1^{r1}$ and $Dsor1^{r2}$, by P-insertional mutagenesis as described in Experimental Procedures. A Southern blot analysis revealed a single P insertion in the mutant X chromosome. Genetic instability of $Dsor1^{Gp158}$ was tested by introducing the P transposase activity. Presence of $P[ry^{+}\ \Delta 2\text{-}3](99B)$ (Robertson et al., 1988) caused reversion at a frequency of 1.6% (27 of 1652), while reversion of $Dsor1^{Gp158}$ was never observed in its absence (0 of 1300). A Southern blot analysis demonstrated loss of the P sequence in these revertants (data not shown). The results indicate that the P element is integrated into the *Dsor1* locus.

A 12 kb BamHI fragment containing the P sequence and its wild-type genomic fragments was cloned. Restriction maps of these cloned fragments revealed an insertion of an incomplete P of 2.6 kb into a 9.6 kb BamHI fragment (Figure 3A). The 9.6 kb BamHI fragment hybridized to the 8D region on the salivary gland polyten chromosome, as predicted from the genetic map position of *Dsor1* (data not shown). cDNA cloning and RNA blot studies using the 9.6 kb BamHI fragment identified three transcription units in this region, in one of which the P element was inserted. To analyze which transcript is affected by the P insertion, the $Dsor1^{Gp158}$ chromosome was marked with *yellow* (*y*), and RNA was extracted from the *y* $Dsor1^{Gp158}/Y$ larvae. An RNA blot analysis revealed that only the transcription unit with the P insertion was affected. A probe upstream of the P integration site detected transcripts of 4–5 kb instead of

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Dsor1  MSKNKLN-LVLPPVNTEATVAAATVAPTPPFKTPSGTDLLGKPKTSIDALTETLEGLDMGDTERKRIKMFLSQKEKIGELSDEDLEKLGE   89
MEK    MPKKKPTPIQLNPAPDGSAV--------------NGTSSA---ETNLEALQKKLEELELDEQQRKRLEAFLTQKQKVGELKDDDFEKISE   73

Dsor1  LGSGNGGVVMKVRHTHTHLIMARKLIHLEVKPAIKKQILRELKVLHECNFPHIVGFYGAFYSDGEISICMEYMDGGSLDLILKRAGRIPE  179
MEK    LGAGNGGVVFKVSHKPSGLVMARKLIHLEIKPAIRNQIIRELQVLHECNSPYIVGFYGAFYSDGEISICMEHMDGGSLDQVLKKAGRIPE  163

Dsor1  SILGRITLAVLKGLSYLRDNHAIIHRDVKPSNILVNSSGEIKICDFGVSGQLIDSMANSFVGTRSYMSPERLQGTHYSVQSDIWSLGLSL  269
MEK    QILGKVSIAVIKGLTYLREKHKIMHRDVKPSNILVNSRGEIKLCDFGVSGQLIDSMANSFVGTRSYMSPERLQGTHYSVQSDIWSMGLSL  253

Dsor1  VEMAIGMYPIPPPNTATLESIF-----ADNAEESGQPT----------DEPRAMAIFELLDYIVNEPPPKLEHKIFSTEFKDFVDICLK  343
MEK    VEMAVGRYPIPPPDAKELELLFGCHVEGDAAETPPRPRTPGRPLSSYGMDSRPPMAIFELLDYIVNEPPPKLPSGVFSLEFQDFVNKCLI  343

Dsor1  KQPDERADLKTLLSHPWIRKAELEEVDISGWVCKTMDL-PPSTPKRNTSPN                                        393
MEK    KNPAERADLKQLMVHAFIKRSDAEEVDFAGWLCSTIGLNQPSTPTHAASI                                         393
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Figure 3. The *Dsor1* Gene

(A) Restriction maps around the P element integration site on the *Dsor1*^{Gp158} chromosome. The arrow indicates the P element. Above the restriction map, the λ phage clones with overlapping inserts are indicated. Positions and orientations of the three transcription units are indicated below the restriction maps. The exon–intron structure is shown only for *Dsor1*. Restriction sites: B, BamHI; G, BglII; H, HindIII; P, PstI; R, EcoRI; S, SalI; and X, XbaI.

(B) The amino acid sequence of Dsor1 predicted from the genomic and cDNA sequences of *Dsor1* is aligned with that of murine MEK (Crews et al., 1992). Hyphens indicate insertions introduced to maximize similarity, and identical residues are indicated with asterisks. The kinase domain is boxed by a dotted line. The amino acid residues are identical at 66.1% of the positions in the entire molecule and 73.5% in the kinase domain.

(C) *Dsor1* is expressed throughout development. Poly(A)$^+$ RNAs from 0–1 hr embryos (lane 1), 0–11 hr embryos (lane 2), first instar larvae (lane 3), second instar larvae (lane 4), third instar larvae (lane 5), early pupae (lane 6), late pupae (lane 7), male flies (lane 8), and female flies (lane 9) were transfered to a nitrocellulose filter after separation in an agarose gel and hybridized with a cDNA probe. The same filter was exposed for 13 hr (top) or 3 days (bottom). The arrow indicates the position of 18S ribosomal RNA (2 kb).

the normal transcript of 2.0 kb, but no transcript was detected with a downstream probe (data not shown).

The nucleotide sequences of both genomic and cDNA fragments revealed the structure of the *Dsor1* gene with three introns, specifying a protein of 393 amino acid residues with an estimated molecular weight of 43,542 (Figure 3B). The nucleotide sequence of *Dsor1*^{Gp158} identified the P element insertion at nucleotide 1229 in the second exon.

An RNA blot analysis revealed that a transcript of approximately 2.0 kb is expressed throughout development and a large number of transcripts present in the early embryos, suggesting a maternal contribution (Figure 3C). The expression pattern is essentially the same as observed with *D-raf* (Nishida et al., 1988).

Dsor1 Encodes a Kinase Similar to MAP Kinase Activator

A search of the National Biomedical Reseach Foundation protein data base demonstrated considerable similarities of Dsor1 to a number of protein serine/threonine kinases. Comparison of the amino acid sequences in the kinase domain demonstrated that Dsor1 is most related to a family of products of the yeast genes PBS2 (Boguslawski and Polazzi, 1987) and STE7 (Teague et al., 1986) of Saccharomyces cerevisiae and byr1 (Nadin-Davis and Nasim, 1988) of Schizosaccharomyces pombe. The identities of the amino acid sequence of Dsor1 is 52.7% with PBS2, 50.1% with byr1, and 47.1% with STE7 in the kinase domain. During preparation of this manuscript, partial peptide sequence of the Xenopus MAP kinase activator (Kosako et al., 1992) and cDNA cloning of the murine MAPK–Erk kinase (MEK) protein (Crews et al., 1992) have been reported, and the sequence of MEK showed a striking similarity to Dsor1 (Figure 3B).

Discussion

A candidate gene for a factor downstream of the D-raf kinase, *Dsor1*, was identified by genetic screening for dominant suppressors of *D-raf*. Its loss-of-function pheno-

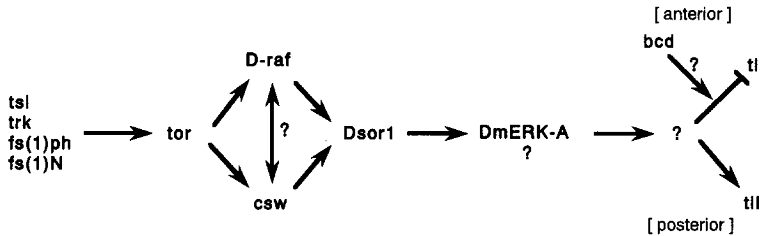

Figure 4. A Model for the Terminal System Signal Transduction Pathway

Primary determination of the body pattern along the anterior–posterior axis of the embryos is carred out by three classes of maternal-effect genes: anterior, posterior, and terminal classes (Nüsslein-Volhard et al., 1987). In the terminal class mutant embryos, nonsegmented anterior and posterior regions (acron and telson, respectively) are deficient (Schüpbach and Wieschaus, 1986; Degelman et al., 1986; Perrimon et al., 1986). Among the terminal class genes, *tor* encodes a receptor tyrosine kinase whose intracellular kinase domain resembles that of the platelet-derived growth factor receptor and the *ret* proto-oncogene product (Sprenger et al., 1989). Epistatic analyses using a gain-of-function allele of *tor* suggested that the products of *tsl* and *fs(1)ph* function upstream of tor (Stevens et al., 1990), while D-raf acts downstream (Ambrosio et al., 1989b). Embryos double mutant between the zygotic genes, *tll* and *huckbein* (*hkb*), showed similar terminal defects, and they would be the target genes of the terminal system (Strecker et al., 1986; Weigel et al., 1990). The expression pattern of *tll* in terminal class mutant embryos may indicate that the signal transduction pathway regulates *tll* positively at the posterior end but negatively at the anterior region (Figure 2). Bcd is involved in the complex expression pattern of *tll* at the anterior region (Pignoni et al., 1992). A nonreceptor protein-tyrosine phosphatase encoded by *csw* also acts downstream of tor in concert with D-raf (Perkins et al., 1992), although the interaction between D-raf and csw is not known. The genetic interactions of *Dsor1* with these terminal class genes suggest that Dsor1 acts downstream of D-raf and csw in this cascade. Identification of the product of *Dsor1* as a protein kinase similar to MAP kinase activator predicts a MAP kinase–like molecule further downstream of Dsor1, and DmERK-A may be the candidate (Biggs and Zipursky, 1992). A transcription factor(s) responsible for the expression of *tll* and *hkb* is still under research.

types and the genetic interactions with *D-raf* and terminal class genes are consistent with the assumption that Dsor1 acts downstream of D-raf. The molecular cloning of *Dsor1* identified its gene product as a protein kinase with a remarkable similarity to the yeast *PBS2*, *STE7*, and *byr1* gene products (Boguslawski and Polazzi, 1987; Nadin-Davis and Nasim, 1988; Teague et al., 1986). The recently cloned murine *MEK* cDNA predicts a primary structure of a polypeptide with a striking similarity to Dsor1 (Crews et al., 1992), indicating that Dsor1 is a Drosophila homolog of the MAP kinase activator.

Both Raf-1 and MAP kinases (or extracellular signal-regulated protein kinases [ERKs]) are involve in transduction of growth-stimulating transmembrane signals (for reviews see Li et al., 1991; Rapp, 1991; Pelech and Sanghera, 1992). It has been shown that MAP kinases phosphorylate and activate Raf-1 (Anderson et al., 1991; Lee et al., 1992), but recent biochemical observations suggested an opposite cascade direction: Raf-1 activates the MAP kinase activator (Kyriakis et al., 1992; Howe et al., 1992). Our genetic results are consistent with the latter observations and provide the first evidence that the raf kinase acts upstream of the MAP kinase activator in physiological conditions. As the raf kinase and MAP kinase activator are highly conserved between Drosophila and vertebrates, a MAP kinase homolog(s) would be expected further downstream of Dsor1. DmERK-A, a Drosophila homolog of ERK recently identified by molecular cloning, may be the candidate (Biggs and Zipursky, 1992).

The genetic interactions observed in this study do not prove a direct interaction between D-raf and Dsor1. It has been shown that the STE7 kinase responsible for activating FUS3 and KSS1, which are close relatives to vertebrate MAP kinases, is under the control of yet another kinase, STE11, in the pheromone response pathway in budding yeast (Marsh et al., 1991; Gartner et al., 1992; Stevenson et al., 1992; Cairns et al., 1992). The byr2 and byr1 kinases related to STE11 and STE7, respectively, are involved in the homologous pathway in fission yeast (Nadin-Davis and Nasim, 1988; Wang et al., 1991). A homolog(s) of STE11 and byr2 might mediate signals from D-raf to Dsor1. The roles and significance of such a cascade of protein kinases remain to be solved.

The multifunctional nature of *D-raf* indicates that the D-raf kinase is involved in a variety of transmembrane signaling processes associated with not only cellular proliferation but also cellular differentiation. As $Dsor1^{Su1}$ suppressed the *D-raf* mutant phenotypes, Dsor1 would function in these processes as well. The best-characterized pathway is the one for the determination of cell fates at the embryonic termini, and a model for the signaling cascade is illustrated in Figure 4. It should be noted that the role of the terminal system on *tll* differs between the anterior and posterior regions of the embryos. The anterior pattern of the *tll* expression is under the control of the terminal system and an anterior class gene, *bicoid* (*bcd*) (Pignoni et al., 1992). The action of the terminal system on *tll* could be altered by an interaction with bcd or factor(s) under the control of bcd, and this combined mechanism would be of biological significance to increase the diversity of responses to a stimulus. $Dsor1^{Su1}$ did not affect the anterior defects of the terminal class mutant embryos. This could be simply explained by the little effect of the gain-of-function mutation on the negative role of the terminal system at the anterior region, although other explanations involving complex interactions of the terminal system with bcd would also be possible. Identification of the transcription factor(s) downstream of Dsor1 is necessary to elucidate the mechanism for the regulation of *tll* and the effects of $Dsor1^{Su1}$ on *tll*. The D-raf and Dsor1 kinases are also required in the pathway regulating cellular proliferation. The receptor(s) and its ligand(s) in this pathway are yet to be identified.

The D-raf and Dsor1 kinases seem to be employed as common signal transducers in a variety of ligand-receptor systems. In addition to *tor*, we observed a significant suppression of *faint little ball* (*flb*), a mutation of the Drosophila epidermal growth factor receptor homolog gene (Price et

al., 1989; Schejter and Shilo, 1989), and *sevenless* (*sev*) (Basler and Hafen, 1988) by *Dsor1*^{Su1} (Y. H. I. and Y. N., unpublished data). This implies complex regulatory mechanisms in which ligand-receptor systems induce effector functions specific to each system in spite of common cytoplasmic factors in between. Drosophila would provide an excellent system to unravel such complex networks of signal transduction, and mutants obtained in the present study would be useful for these studies.

Experimental Procedures

Genetics

Fly culture and crosses were performed according to standard procedures. Fly stocks used in this study were provided as follows: *D-raf*^{C110}/*FM7* from N. Perrimon; *tor*^{PM} *cn bw/CyO*, *tor*^{RL3} *cn px sp/CyO*, *tsl*^{p35} *e/TM2*, *trk*^{RA41} *cn bw/CyO*, *fs(1)ph*^{1901}/*FM6*, and *tll*^{1} *e/TM2* from G. Struhl; *Fs(1)ovo*^{D1} *v/C(1)RX*, *y f/B*^{S}Y from A. P. Mohowald; *lz/FM6a/lz*$^{+}$*dor*$^{+}$Y from M. Yamamoto; and *E(sev)1A*^{e0P} *sev*^{d2}/*FM7* from G. M. Rubin and M. A. Simon. For descriptions of the genetic markers and balancers, see Lindsley and Zimm (1992).

For screening of dominant suppressors of *D-raf*, *y D-raf*^{C110}/*B*^{S}*y*$^{+}$*w*$^{+}$Y males were fed 0.01 M EMS and mated to virgin *C(1)RX*, *y f*/Y females. The *B*^{S}*y*$^{+}$*w*$^{+}$Y chromosome carries a portion of X chromosome including the *D-raf*$^{+}$ locus so that the *y D-raf*^{C110}/*B*^{S}*y*$^{+}$*w*$^{+}$Y males are viable. The sons of the above crossing would be lethal because the Y chromosome is replaced with the maternally derived normal Y chromosome.

To isolate revertants of *Dsor1*^{Su1}, *y D-raf*^{C110} *sn Dsor1*^{Su1}/Y males were fed 0.01 M EMS and crossed to *Df(1)Pgd-kz/FM7c* females, and their *y D-raf*^{C110} *sn Dsor1*^{Su1} (mutagenized)/*FM7c* daughters were individually crossed to five *FM7c*/Y males. Among about 3700 crosses, 328 recessive lethals were obtained. They were further tested by mating the females with the EMS-treated chromosomes balanced over *FM7c* to *y D-raf*^{C110} *sn/B*^{S}*y*$^{+}$*w*$^{+}$Y males to determine whether the lethality is due to the reversion of *Dsor1*^{Su1} or to additional lethal mutations. The females with the genotype of *y D-raf*^{C110} *sn Dsor1*^{Su1}(mutagenized)/*y D-raf*^{C110} *sn* would be lethal if *Dsor1*^{Su1} were reverted.

To induce a P element insertional mutation at the *Dsor1* locus, we used the Birmingham 2 stock with 17 nonautonomous P elements on the second chromosome as a P source and *P[ry*$^{+}$ *Δ2-3](99B)* as the source of the P transposase (Robertson et al., 1988). *Birm2/Birm2; Sb/TM6, Ubx* females were crossed with *Cy/Sp;P[ry*$^{+}$ *Δ2-3](99B)/P[ry*$^{+}$ *Δ2-3](99B)* males, and their dysgenic males were mated to *Df(1)Pgd-kz/FM7c* females. The daughters with the mutagenized X chromosome balanced over *FM7c* and with dominant markers on the second and third chromosomes were individually mated with five *FM7c*/Y males. Among about 18,000 crosses, 171 X-linked recessive lethals were obtained, and they were further tested for the allelism to *Dsor1* by crossing the heterozygous females with *Dsor1*^{r1}/*lz*$^{+}$*dor*$^{+}$Y or *Dsor1*^{r2}/*lz*$^{+}$*dor*$^{+}$Y males. A portion of X chromosome including *Dsor*$^{+}$ is translocated on *lz*$^{+}$*dor*$^{+}$Y. One recessive lethal that failed to complement *Dsor1*^{r1} and *Dsor1*^{r2} was obtained.

Mosaic Analysis

Dp(1;Y;3)M2, *mwh*$^{+}$ *ve*$^{+}$ *FR1*, *y cv v f/FM7a/B*^{S}*Y;mwh ve h* stock was provided by A. Garcia-Bellido, and *f* was replaced with *f*^{36a} by genetic recombination to make a stock, *Dp(1;Y;3)M2'*, *mwh*$^{+}$ *ve*$^{+}$ *FR1*, *y cv v f*^{36a}/*C(1)RX*, *y f/B*^{S}*Y;mwh ve h*. The third chromosomes of all the tested stocks were marked with *multiple wing hairs* (*mwh*), and clonal analysis was performed on twin spots formed in wings as described (Postlethwait, 1978; Lawrence et al., 1986). Only the twin spots with more than 16 *f*^{36a} (control) cells were taken into account. Germline mosaics were produced by using *Fs(1)ovo*^{D1} according to Perrimon et al. (1985).

Molecular Analysis

The DNA from *Dsor1*^{Gp158}/*FM7c* females was digested with BamHI, and fragments of 10–15 kb purified from agarose gels were ligated with EMBL3 BamHI arms. Ligated DNA was in vitro packaged, and the constructed library was screened with a P element probe that has been polymerase chain reaction amplified by using the 31 bp terminal inverted repeat of P as a primer and pπ25.1 plasmid DNA as a template. The wild-type genomic fragments were cloned from an EMBL3 library containing Oregon R fly DNA partially digested with SauIIIAI. cDNA libraries from embryos constructed in the λgt10 vector (Poole et al., 1985) and the pNB40 vector (Brown and Kafatos, 1988) were provided by L. M. Kauvar and N. H. Brown, respectively. For DNA sequencing, the genomic and cDNA fragments were cloned into pGEM-3 (Promega), and clones with a series of deletions were produced using the Deletion Kit for Kilo-Sequencing (Takara Shuzo Company, Japan). DNA sequences were determined by using the Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems) with SP6 and T7 primers and analyzed with the Model 373A DNA Sequencing System (Applied Biosystems). The DNA sequences at the P integration site in *Dsor1*^{Gp158} were determined by using internal P element primers and the cloned 12 kb BamHI fragment as a template. Other molecular procedures are as described (Sambrook et al., 1989).

Other Procedures

Cuticle preparations of embryos were made as described (Wieschaus and Nüsslein-Volhard, 1986). In situ hybridization on whole embryos was performed as described (Tautz and Pfeiffle, 1989) using the 1.9 kb insert of the *tll* cDNA clone N4 (Pignoni et al., 1990), which was digested with AluI before labeling with digoxigenin.

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