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Dissection of the Torso Signal Transduction Pathway in *Drosophila*

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ABSTRACT Cell fate choice at the anterior and posterior embryonic termini of the *Drosophila* embryo requires the activation of a signal transduction pathway regulated by the receptor tyrosine kinase Torso. When Torso, which is uniformly distributed in the egg cell membrane, becomes activated locally at the termini, it triggers a phosphorylation cascade that culminates with localized expression of the transcription factors, *tailless* and *huckebein*. Expression of *tailless* and *huckebein* in turn determines terminal cell fates. Several genes have been characterized which encode proteins that are involved in Torso signaling: the adaptor protein Drk, the GTP-binding protein Ras1, the guanine nucleotide exchange factor Son of sevenless, and the kinases D-Raf and D-Mek. Genetic and molecular evidence supports a model in which these proteins lie in the same biochemical pathway. When activated by its ligand the membrane-bound receptor tyrosine kinase Torso initiates a signal transduction pathway mediated by Drk, Sos, and Ras1, which in turn activates a phosphorylation cascade mediated by the kinases D-Raf and D-Mek, which ultimately control the localized expression of the transcription factors *tailless* and *huckebein*. Recently, we found that D-Raf can be partially activated by Torso in the absence of Ras1, a finding supported by the phenotype of embryos lacking either Drk or Sos activity, as well as by the phenotype of a *D-raf* mutation that abolishes binding of Ras1 to D-Raf. These findings indicate that full D-Raf activation requires input not only from Ras1 but also from an as yet uncharacterized Ras1-independent pathway. In addition to these molecules we have characterized the putative protein tyrosine phosphatase Corkscrew as a positive transducer downstream of Torso.

Key Words: Receptor tyrosine kinase, Ras, Raf, MEK, *Drosophila*

INTRODUCTION

Studies on receptor tyrosine kinase (RTK) signaling pathways in both vertebrates and invertebrates have converged on an evolutionarily conserved cassette of genes that are required for transducing the signal from the plasma membrane to the nucleus. While biochemical analyses in mammalian cells have defined the interactions between molecules that respond to activation of RTKs, genetic studies in *Caenorhabditis elegans* and *Drosophila* have defined the in vivo requirements for these molecules in signaling. The combined knowledge obtained from both approaches has provided a comprehensive view of the sequence of events following binding of a ligand to an RTK (review by Perrimon and Desplan, 1994).

Following ligand binding, RTK molecules undergo dimerization, inducing autophosphorylation of specific tyrosine residues on the cytoplasmic domains of the receptor (review by van der Geer et al., 1994). These phosphotyrosines serve as "docking sites" for cytoplasmic proteins which bind to the activated RTK through their SH2 domains. One of these SH2-containing proteins is known in *Drosophila* as Drk (also known as Sem 5 in *C. elegans* and Grb2 in vertebrates). Drk consists of one SH2 and two SH3 domains and serves as an adaptor protein to recruit, via one of its SH3 domains, the $p21^{ras}$-exchange factor encoded by the *Son of Sevenless (Sos)* gene. Translocation of Sos to the membrane facilitates the conversion of inactive GDP-Ras1 to activated GTP-Ras1 which further transduces the signal through the D-Raf, D-Mek, and Rolled (MAPK) kinase cascade. Activated MAPK is translocated to the nucleus where it modifies the activity of a subset of transcription factors.

Our laboratory is studying the Torso RTK signal transduction pathway. From the pioneer work of Nüsslein-Volhard and her colleagues (see reviews by St. Johnston and Nüsslein-Volhard, 1992; Duffy and Perrimon, 1994; Fig. 1), three different systems have been identified that control cell fates along the anteroposterior axis of the embryo. The anterior, or Bicoid, system controls pattern formation of the thorax and part of the head; the posterior, or Nanos, system controls pattern formation of the abdomen; and the terminal, or Torso, system controls formation of the tail and the most anterior parts of the head. The three patterning systems ultimately control the expression of unique

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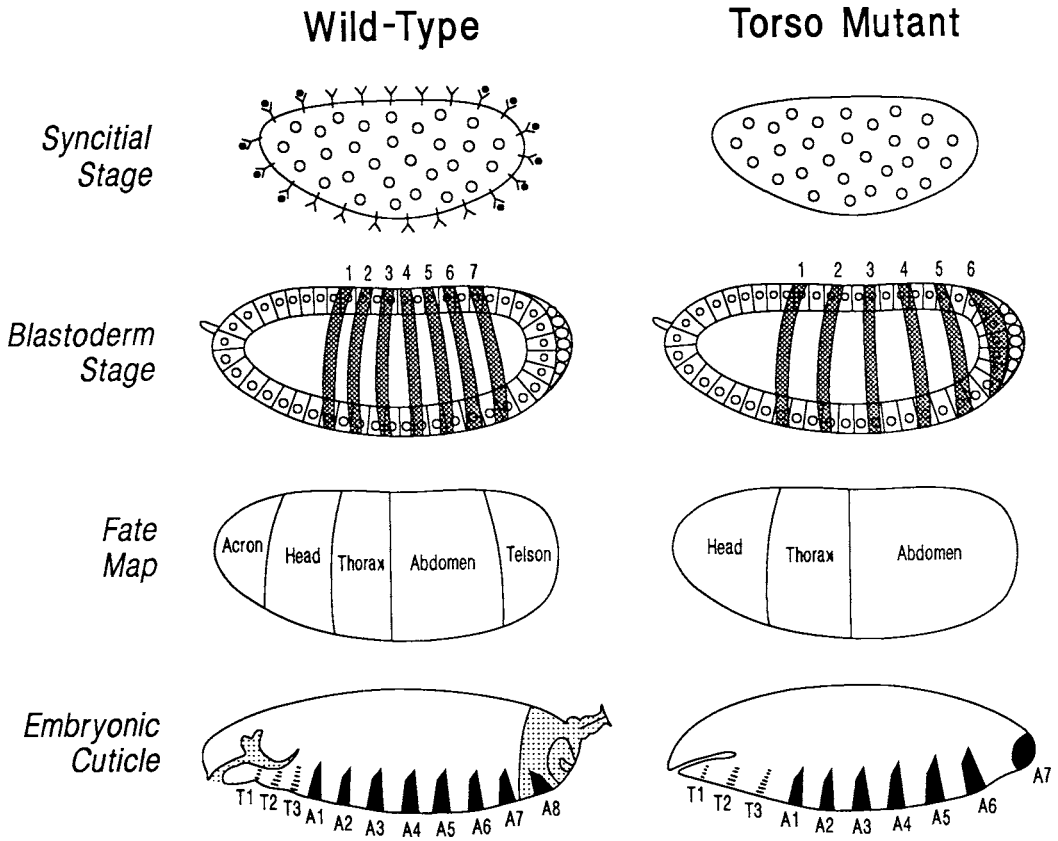

Fig. 1. Torso signaling during embryogenesis. In the wild-type embryo, the terminal or Torso system operates along the anterior and posterior axes, in combination with the anterior and posterior systems, to define cell fates. The Torso RTK, which is locally activated at each terminus by an activity present in the perivitelline fluid, triggers a signal transduction pathway that controls gene expression at the termini. At the cellular blastoderm stage the overall body plan, as shown by the expression of the pair rule gene *fushi-tarazu (ftz)* in seven stripes, is already determined. In a *torso* mutant, the signaling pathway triggered by Torso RTK activation does not operate resulting in the deletion of terminal regions and expansion of the embryonic domains specified by the anterior and posterior systems. The terminal cuticular regions that include part of the head skeleton and all structures posterior to abdominal segment 7 are deleted in *torso* mutant animals. (Reproduced with permission from Perrimon, 1993.)

sets of transcription factors. Two genes encoding putative transcription factors regulated by Torso are *tailless*, a member of the steroid hormone receptor superfamily, and *huckebein*, a zinc finger containing transcription factor. Both *tailless* and *huckebein* are expressed in the posterior and anterior regions of the embryo. Posterior expression of these genes is under the control of the Torso pathway where in *torso* loss of function mutant animals they fail to be expressed, and conversely, in torso gain of function mutants their domains of expression are expanded. Anterior expression of these genes is more complicated since their expression requires input not only from Torso but also from the Bicoid patterning system (Ronchi et al., 1993).

Researchers from a number of laboratories (see review by Duffy and Perrimon, 1994) have proposed a model that describes how the transcription factors *tailless* and *huckebein* become expressed at the termini. Torso, which is uniformly expressed in the egg, becomes locally activated by a ligand confined to each terminus. A putative candidate for the Torso ligand is encoded by the gene *torso-like* which encodes a novel secreted protein. Local activation of Torso at the egg poles then triggers the signal transduction pathway that activates *tailless* and *huckebein*.

The Torso signaling pathway generates a graded activity that is revealed in the wild-type cellular blastoderm by the overlapping domains of expression of *tailless* and *huckebein* which are solely dependent upon the Torso signal. Posteriorly, *huckebein* is expressed in a smaller domain than *tailless*. The differences between these two posterior domains of expression reflect the differential responses of the *tailless* and *huckebein* promoters to the strength of the Torso signaling pathway. Therefore the spatial domains of *tailless* and *huckebein* expression can be used as read outs for the strength of the Torso signal transduction cascade. This is a critical feature of this pathway since it allows us to directly monitor the strength of the signaling cascade by monitoring the domains of expression of *tailless* and *huckebein*.

ROLE OF D-RAF IN TORSO SIGNALING

Searches for maternal effect mutations that perturb the organization of the anteroposterior axis have led to

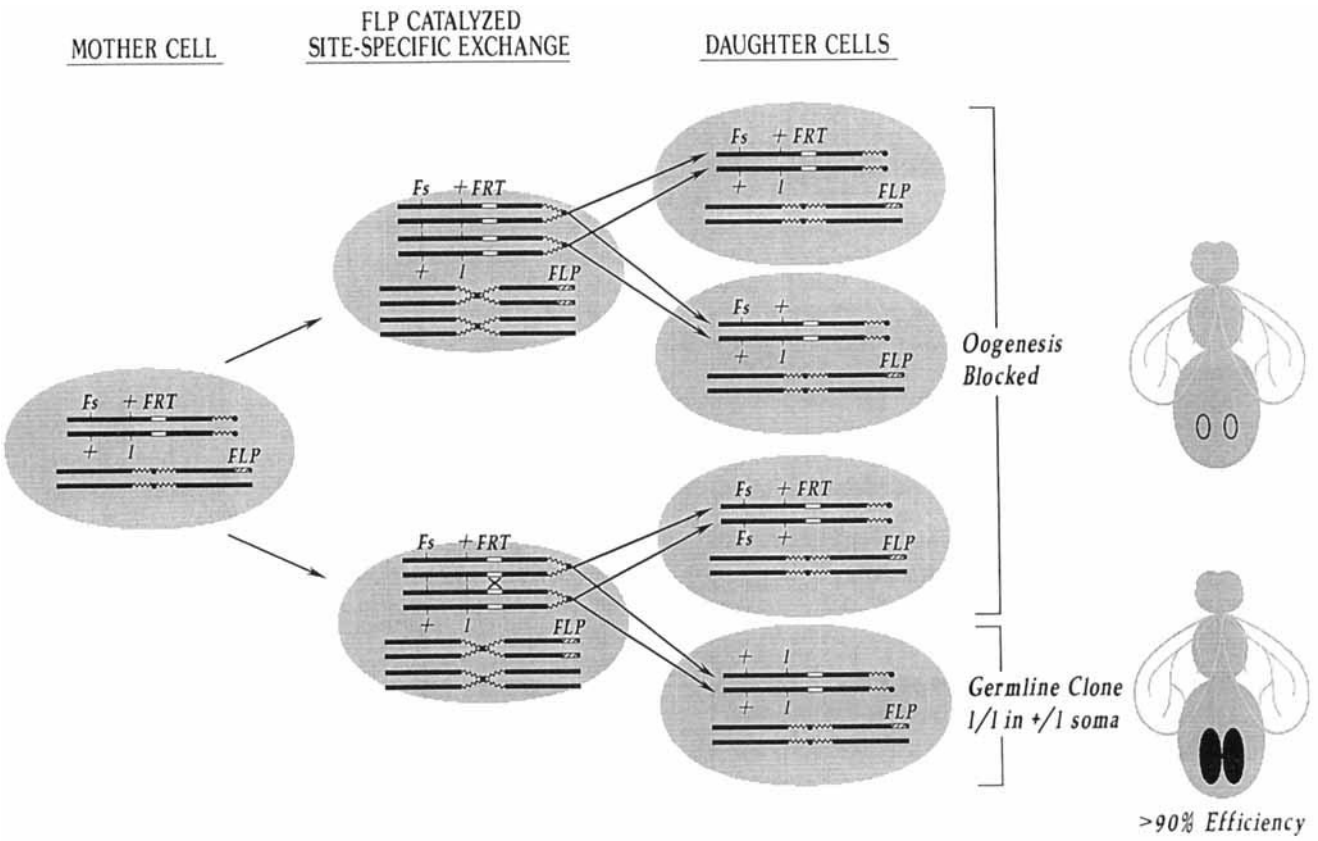

Fig. 2. The "FLP-DFS" technique. An efficient method for generating female germline mosaics by inducing site-specific homologous mitotic recombination with a yeast recombinase has been developed by Chou and Perrimon (1992). These germline mosaics are produced in flies heterozygous for the agametic, germline-dependent dominant female sterile mutation ovo^{D1} (Fs) where only flies possessing germline clones are able to lay eggs. Females heterozygous for ovo^{D1} never lay eggs because they have rudimentary ovaries in which oogenesis is blocked at an early stage. The female sterility associated with the ovo^{D1} mutation is strictly germline dependent such that flies carrying this mutation are perfectly viable and wild-type germ cells in an ovo^{D1} heterozygous female produce wild-type eggs. In addition, germ cells heterozygous for ovo^{D1} develop poorly so that when a germ cell has eliminated ovo^{D1} it has a growth advantage and the resulting clone can usually give rise to a fully developed ovary. This overproliferation of wild-type cells in an ovo^{D1} background has been a major advantage in generating germline mosaics. To generate a high frequency of mitotic recombination events in females heterozygous for ovo^{D1}, we have taken advantage of the properties of the yeast "FLP-FRT" site-specific recombination system. The yeast FLP-recombinase and its recombination targets (FRTs) from the 2 μm plasmid of *Saccharomyces cerevisiae* were successfully transferred into the *Drosophila* genome (Golic and Lindquist, 1989). In this system, the heat-inducible FLP-recombinase gene, under the control of an *hsp70* promoter, recognizes and promotes recombination specifically at the level of the FRT sequences. This method, the "FLP-DFS" technique, is very efficient since more than 90% of females with germline clones can be recovered. This technique has been extended to the autosomes (Chou et al., 1993; Chou and Perrimon, in preparation). The principle of the FLP-induced site-specific exchange is depicted in this figure where a chromosomal exchange that occurs in the euchromatin of a fly of genotype *Fs + FRT/+ l FRT;FLP/FLP* is shown. In this scheme, the FRT insertion is located proximally to both Fs and *l* on the X chromosome. The autosomally located *hsp70-FLP* can provide sufficient recombinase activity following heat induction to catalyze site-specific chromosomal exchange at the position of the FRT sequences. FLP-catalyzed recombination can result in the recovery of greater than 90% of females with *l/l* germline clones. Atrophic ovaries are shown as empty ovals and developed ovaries as black ovals. Flp-recombinase target sequences (FRT) are depicted as blank boxes and FLP as stippled boxes. Dominant female sterile (Fs), recessive zygotic lethal mutation (l), hsp70-Flp (FLP). (Reproduced with permission from Chou and Perrimon, 1992.)

the identification of a number of genes that operate in the Torso pathway (see reviews by St. Johnston and Nüsslein-Volhard, 1992; Duffy and Perrimon, 1994). The approach our laboratory has used is to identify mutations that cause embryonic terminal defects among late zygotic lethal mutations using techniques of germline mosaics (Perrimon et al., 1989).

Genetic evidence indicates that the *Drosophila* genome contains about 5,000 mutable genes. Of these 5,000 genes, approximately 85% are mutable to zygotic lethality. Normal gene activity of at least 75% of these essential genes is required during oogenesis (Perrimon et al., 1989) which is consistent with molecular analyses, which show an extensive store of maternal mRNAs in the unfertilized egg and that nearly all of the mRNA complexity at blastula and gastrula stages is represented in the egg mRNA. The use of germline clonal analyses (Perrimon et al., 1989; Chou and Perrimon, 1992; Fig. 2) has allowed us to analyze the maternal effect phenotypes of zygotic lethal mutations. From these screens we have identified two novel terminal class genes, *l(1)pole hole* and *corkscrew* (Perrimon et al., 1989).

l(1)pole hole mutations have a maternal effect phenotype that is identical to *torso* mutations, and further, they suppress the dominant phenotype associated with a *torso* gain of function mutation suggesting that *l(1)pole hole* acts downstream of Torso (Ambrosio et al., 1989). Molecular characterization of *l(1)pole hole* showed that it encodes a protein serine/threonine protein kinase with 46% identity at the amino acid level to the human *Raf-1* kinase (Melnick et al., 1993). *l(1)pole hole* encodes the *Drosophila* homolog of mammalian Raf1 since the mammalian Raf protein can rescue all of the defects associated with a *l(1)pole hole* loss of function mutant. We therefore now refer to *l(1)pole hole* as *D-raf* to simplify the nomenclature. The D-Raf protein has three highly homologous domains in common with the mammalian protein: the CR1 domain, implicated in binding to Ras-GTP and the 14-3-3 proteins; the CR2 domain, implicated in binding the 14-3-3 proteins; and the CR3 domain, the serine/threonine kinase catalytic domain (reviews by Daum et al., 1994; Morrison, 1994; Morrison, this volume).

D-RAF CAN BE ACTIVATED BY TORSO IN THE ABSENCE OF DRK, SOS, OR RAS1

To identify additional molecules involved in the transduction of the Torso signal, we decided to test whether the small GTPase protein Ras1 played a role in D-Raf regulation. Indeed previous studies in mammalian cells had provided evidence that activated Ras could regulate Raf activity (see review by Egan and Weinberg, 1993). To test the possibility that Ras1 could be acting upstream of D-Raf to transmit information from the Torso RTK, we injected the dominant negative protein $p21^{rasN17}$, which blocks the activity of the Ras exchange factor, into wild-type precellular embryos (Lu et al., 1993). If $p21^{rasN17}$ blocks the signal from Torso then the developing embryos should exhibit terminal defects. Results from these experiments demonstrated that indeed $p21^{rasN17}$ can block the activity of Torso, however the Torso signal was not completely blocked.

To further understand the roles of proteins involved in Ras1 regulation in Torso signaling we directly analyzed the maternal effect phenotypes of mutations in *Drk*, *Ras1*, *Sos*, and *Gap1* (Hou et al., 1995). In Ras1 regulation, Drk promotes the recruitment of the Ras1-exchange factor Sos to the membrane. Gap1 is a negative regulator of Ras1 since it encodes a Ras-Gap protein that presumably increases the endogenous GTPase activity of Ras1. We generated embryos that completely lacked Ras1 activity (Fig. 3). Using the technique of germline mosaics described in Figure 2, we found that embryos which lacked Ras1 activity showed reduced *tailless* expression consistent with its proposed role in Torso signaling. However, unlike *torso* or *D-Raf* mutant animals, *tailless* was still expressed in *Ras1* mutant embryos, suggesting that activation of *D-raf* requires additional input from a Ras1-independent pathway initiated downstream of the Torso RTK (see Fig. 5).

To further substantiate this observation, we examined the effects on *tailless* expression of deleting the maternal contributions of other members of the Ras1 regulatory pathway. Embryos lacking either Sos or Drk maternal activities had phenotypes similar to that of loss of Ras1. On the other hand, as predicted by its role as a negative regulator of Ras1 activity, embryos that develop from germlines that lack *Gap1* activities show an expansion of *tailless* expression.

Additional evidence for the existence of a Ras1-independent pathway was obtained from the analysis of a mutation in *D-raf*, $D\text{-}raf^{C110}$, which prevents the binding of Ras1-GTP to D-Raf (Hou et al., 1995). $D\text{-}raf^{C110}$ is a weak mutation of *D-raf* in which Torso signaling is not blocked (Melnick et al., 1993). $D\text{-}raf^{C110}$ contains a single point mutation, changing the arginine at position 217 into a leucine. Using the yeast two-hybrid system we were able to demonstrate that the CR1 domain of D-Raf is not able to interact with Ras1 in the presence of the $D\text{-}raf^{C110}$ mutation (Fig. 4). A similar result was obtained by Fabian et al. (1994) who tested the effect of the same mutation on the interaction between the mammalian Raf1 and $p21^{ras}$ proteins. These results are consistent with the existence of a Ras1-independent pathway that leads to the partial activation of D-Raf.

In summary, our results demonstrate that D-Raf is activated by both Ras1-dependent, as well as Ras1-independent pathways (Fig. 5). There are two mechanisms by which these two pathways could cooperate to provide a full level of D-Raf activation. In the first model, Ras1 regulates the level of D-Raf available at the membrane (see also Stokoe et al., 1994; Leevers et al., 1994) where the Ras1-independent pathway subsequently activates D-Raf. In the second model, the Ras1-dependent and Ras1-independent pathways both independently activate D-Raf. Synergism between the two activating pathways will lead to full activation of D-Raf. Distinguishing between these two models will require the identification of molecules that operate in the Ras1-independent pathway.

CORKSCREW, A PROTEIN TYROSINE PHOSPHATASE WITH SH2 DOMAINS

Loss of function mutations in the nonreceptor protein tyrosine phosphatase (PTPase) Corkscrew affect Torso signaling in a manner similar to Ras1 (Perkins et al., 1992). Mutations in *corkscrew* cause a weak terminal phenotype which is apparent not only in the cuticle of the animals derived from *corkscrew* mutant germlines but also in the spatial expression of *tailless*. In the absence of Corkscrew activity, Torso signaling is not completely blocked. Genetic and developmental analysis of *corkscrew* is consistent with the proposal that Corkscrew is a positive signal transducer that acts downstream of Torso. Specifically, genetic epistasis experiments indicate that *corkscrew* loss of function mutations can suppress the effect of gain of function *torso* mutations.

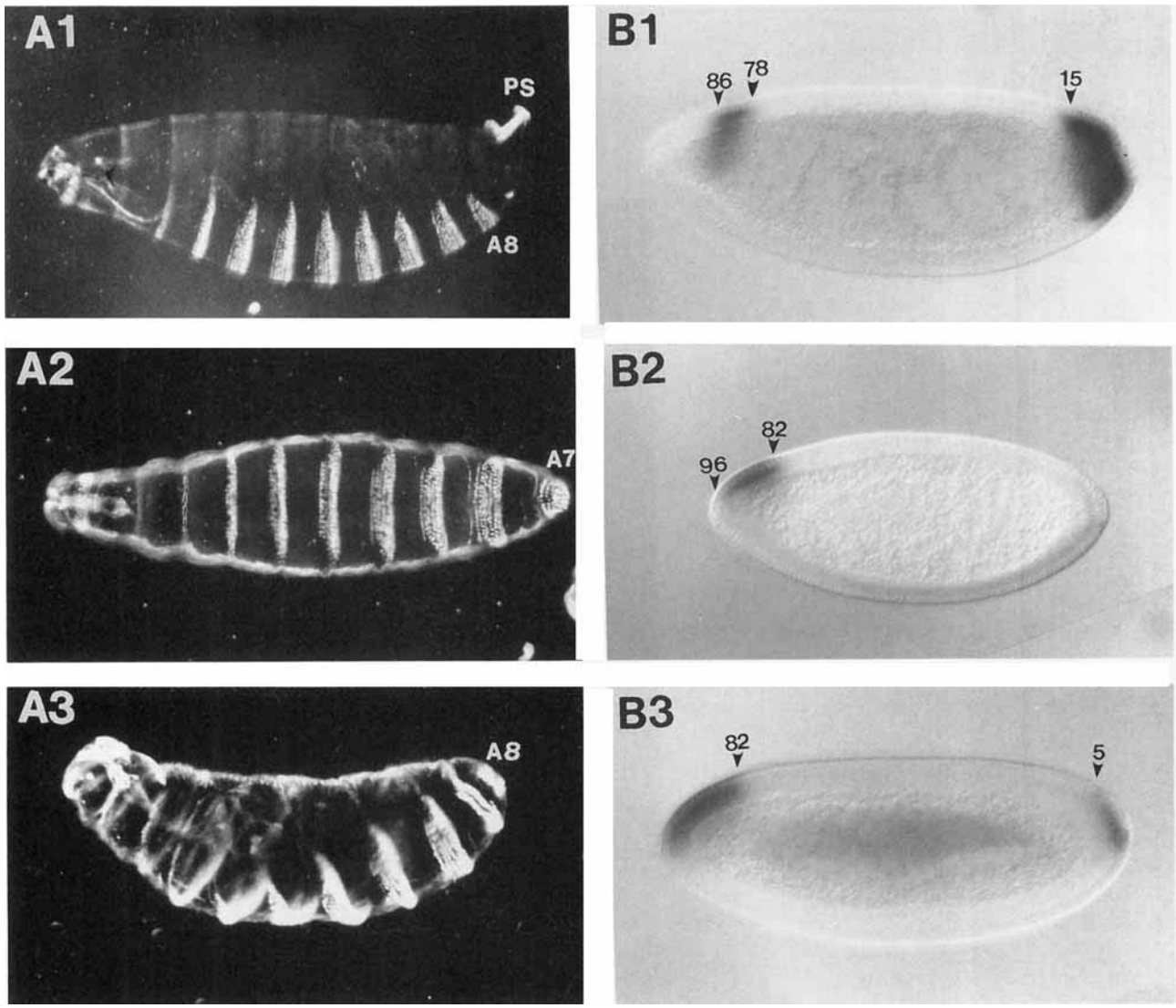

Fig. 3. Phenotypes of *D-raf* and *Ras1* mutant embryos. Cuticle phenotypes of wild-type (**A1**), *D-raf* (**A2**), and *Ras1* (**A3**) embryos. Note that *D-raf* mutant embryos are missing all structures posterior to the seventh abdominal segment, while *Ras1* mutant embryos develop more posterior structures. The expression of *tailless* in wild-type (**B1**), *D-raf* ((**B2**), and *Ras1* (**B3**) mutant embryos is shown. The expression of *tailless* mRNA is visualized by in situ hybridization to cellular blastoderm embryos. All embryos are oriented with the anterior to the left. Note that residual posterior *tailless* expression is detected in embryos that lack *Ras1* activity. Nomenclature: The domain of *tailless* expresssion is indicated as % egg length (EL) by numbers located above the arrow heads. A7 and A8 refer to abdominal segments 7 and 8, respectively.

The Corkscrew protein is similar in structure to the mammalian PTPases SH-PTP2 (also known as PTP2C, PTP1D, and SYP) and PTP1C (also known as SH-PTP1, SHP, and HCP) (Perkins et al., 1992), each of which consists of two N-terminal SH2 domains and a catalytic tyrosine phosphatase domain. In the Corkscrew protein there is an insert of 150 amino acids within the catalytic domain. This insert contains a putative phosphotyrosine and two conserved cysteine fingers that are of interest with respect to its function and regulation. According to the crystal structure of PTP2 (Barford et al., 1994), Corkscrew's insert is located between the second and third β-sheets of the PTPase catalytic domain. When modeled on the structure of PTP2, the Corkscrew insert is located on the opposite side of the PTPase catalytic active site and thus the insert should not directly interfere with the interaction of the catalytic active site and its enzymatic substrates. Accordingly, we find that SH-PTP2, which does not possess an insert, can partially rescue *corkscrew* mutant animals (Melnick and Perkins, unpublished). We conclude that the insert is not absolutely essential for Corkscrew activity.

The position of Corkscrew in Torso signaling is not yet understood. In particular it is unclear whether Corkscrew regulates Ras1 activity or acts on the Ras1-

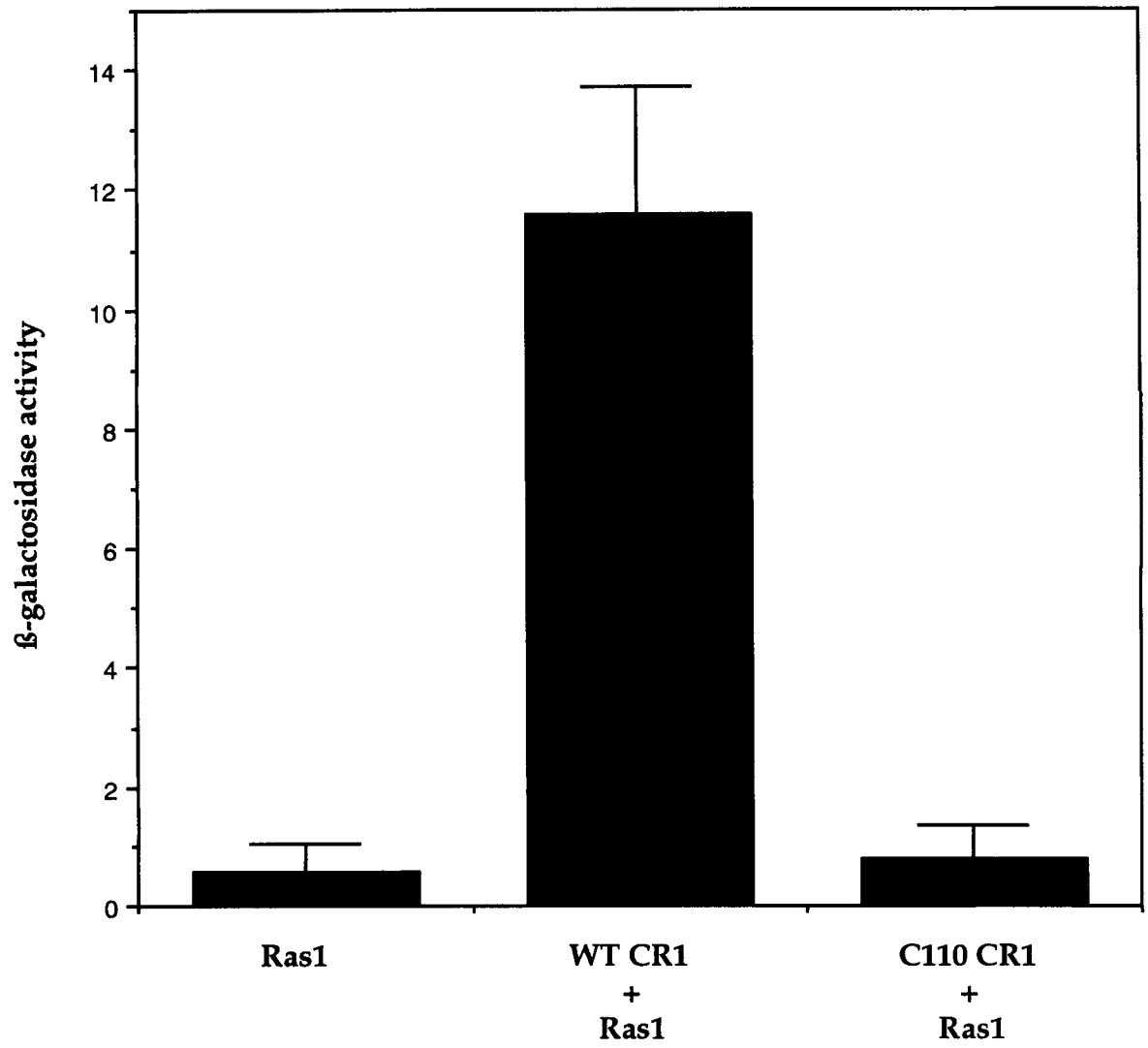

Fig. 4. The *D-raf*^{C110} mutation eliminates binding of Ras1 to D-Raf. The yeast two-hybrid system was used to demonstrate the affinity of Ras1 for the D-Raf CR1 domain and the disruption of this interaction by the *D-raf*^{C110} mutation (R217L). In this analysis we used the CR1 domain of the D-Raf protein rather than full-length D-Raf because the interaction between Ras and the CR1 domain of Raf alone is stronger than between Ras and the full Raf protein (see Vojtek et al., 1993, for example). Affinity between the proteins is reflected by transcription of β-galactosidase, whose activity is given in units defined by Rose et al. (1990). When the *D-raf*^{C110} mutation is introduced, β-gal activity is reduced to the background seen in the presence of Ras1 alone. The D-Raf CR1 domain alone in this assay did not cause measurable activation.

independent pathway to activate D-Raf. It has been proposed that SH-PTP2 acts as an adaptor for Grb2 (Li et al., 1994; Bennett et al., 1994). Consistent with a model whereby Corkscrew functions as an adaptor for Drk in the Ras1-dependent pathway is the observation that the Corkscrew phenotype can be suppressed by injection of activated forms of Ras (Lu et al., 1993). It should be kept in mind, however, that these results do not rigorously establish that Corkscrew is not a component of the Ras1-independent pathway. If two partially activating pathways converge on D-Raf, it is possible that hyperactivation of one pathway can compensate for the loss of the other. Further analyses of the role of Corkscrew is required to establish the function of this PTPase in RTK signaling.

COMPONENTS OF THE TORSO PATHWAY THAT ARE DOWNSTREAM OF D-RAF

To identify components of the Torso signaling pathway that act downstream or upstream of D-Raf we have conducted screens for second site suppressors of the weak *D-raf* mutation *D-raf*^{C110}. *D-raf*^{C110} mutant animals die as late pupae and second site suppressors can be identified by their ability to rescue the *D-raf*^{C110} pupal lethality. From these screens we identified 11 suppressor mutations among which were 4 intragenic

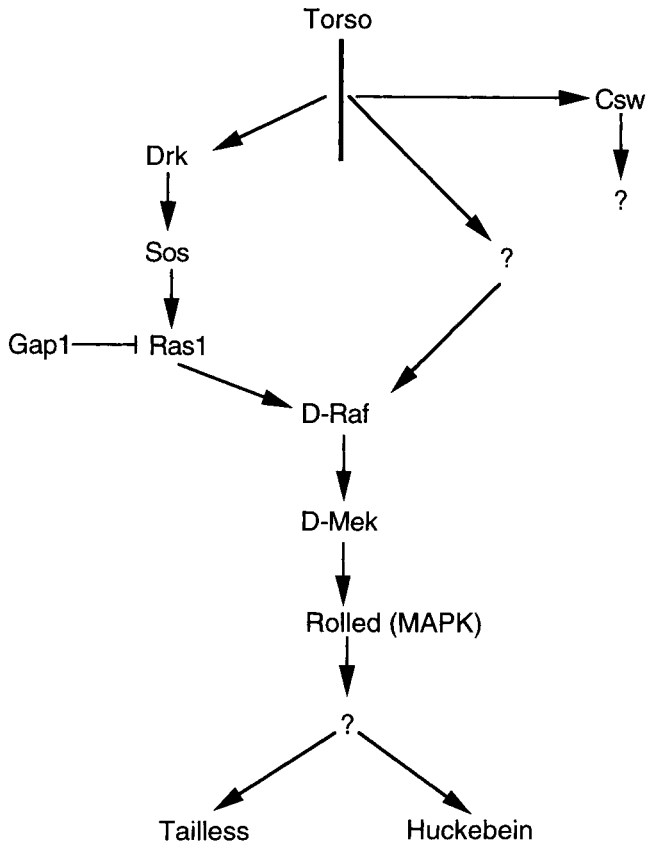

Fig. 5. The Torso signaling pathway (see text).

suppressors in *D-raf* (Lu et al., 1994). Two of them reside within the CR1 domain and two are located in the CR3 or kinase domain of D-Raf. One of the second site suppressors, su34B, mapped to a region of the chromosomes to which a *Drosophila mek* gene had been previously characterized (Tsuda et al., 1993). We found that su34B contained a single amino acid change in D-Mek, which is 65% homologous to the mammalian Mek. An additional second site suppressor was mapped on the second chromosome near the location where the *rolled* MAP kinase gene is located; however, we have not yet determined whether our suppressor actually resides within the *rolled* gene. Two other suppressors, which we have not yet analyzed in detail, map to the third chromosome.

To pursue our genetic characterization of the Torso signaling pathway we have designed additional genetic screens for second site modifiers of a temperature sensitive mutation in *D-mek*. We have generated, by site-directed mutagenesis, a temperature-sensitive *D-mek* allele (Hsu and Perrimon, 1994). A single amino acid change, proline 137 to serine, is associated with a *D-mek* temperature-sensitive phenotype. At 29°C, flies of genotype $D\text{-}mek^{ts}$ exhibit a weak Torso mutant phenotype, but the same flies reared at 25°C and below appear wild type. This unique property of $D\text{-}mek^{ts}$ allows us to screen for second site mutations that either enhance or suppress the $D\text{-}mek^{ts}$ phenotype.

CONCLUSIONS

Our analysis of the *Drosophila* terminal system has provided a paradigm to identify components of an RTK signaling pathway. In addition, genetic and biochemical epistasis experiments have permitted us to characterize the regulatory interactions between the molecules involved in signaling. Currently, by conducting various genetic screens, our efforts are directed towards identifying additional components of the Torso signal transduction pathway. Through genetic analyses we expect to identify components of the Ras1-independent pathway and in this way gain an understanding of how D-Raf is fully activated by an RTK.

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QUESTIONS AND ANSWERS

Q: Does Torso have a PLC-gamma binding site?

A: We do not yet know.

Q: You have studied the effect of microinjecting bacterially derived dominant negative Ras. Have you done the same experiment with dominant negative Ras that was prepared from a baculovirus expression vector so that the recombinant protein is prenylated?

A: This is a good experiment but we have not done it.

Q: Have you prepared the double mutant of Ras 1 and corkscrew to determine whether they are on the same or different pathways?

A: This is a critical experiment to do and I wish I could provide you with the answer. Because *Ras1* and *csw* are not located on the same chromosome we have to construct complicated chromosomes to perform this experiment. This experiment is in progress.