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The role of D-raf in the terminal class signal transduction pathway in Drosophila melanogaster

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The role of D-raf in the terminal class signal transduction pathway in *Drosophila melanogaster*

by

Kwang-Hyun Baek

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INTRODUCTION

The regulation of maternal and zygotic gene expression in Drosophila is required for early pattern formation in the embryo. The embryonic polarity along the anterior-posterior and dorsal-ventral axes of the egg is established through the activity of maternal gene products that accumulate during oogenesis. This activity allows the establishment of specialized domains of zygotic gene expression (Konrad et al., 1985; Mahowald and Hardy, 1985; Akam, 1987; Anderson, 1987; Nüsslein-Volhard et al., 1987; Ingham, 1988).

The Torso Signaling Pathway

It has been shown that three maternal groups of genes are required for the establishment of the anterior-posterior axis (Nüsslein-Volhard et al., 1987; Nüsslein-Volhard, 1991; St. Johnston and Nüsslein-Volhard, 1992). Patterning in the head and thoracic regions is controlled by a morphogenic gradient of the bicoid protein (Driever and Nüsslein-Volhard, 1988a, 1988b). Patterning in the abdominal regions is controlled by a morphogenic gradient of the nanos protein (Wang and Lehmann, 1991). The last group is the terminal class genes and they are required for the development of the head (acron) and tail (telson) (Nüsslein-Volhard et al., 1987; St. Johnston and Nüsslein-Volhard, 1992). The maternally active members in this class have been identified. These include: fs(1)Nasrat (Degelmarm et al., 1986), fs(1)polehole (Perrimon et al., 1986), fs(3)torso-like (Stevens et al., 1990), fs(2)trunk, fs(2)torso (Schüpbach and Wieschaus, 1986), Drk (Olivier et al., 1993; Simon et al., 1993), Sos (Buday and Downward, 1993), D-ras1 (Lu et al., 1993), D-raf [also known as l(1)polehole] (Perrimon et al., 1985; Nishida et al., 1988), D-MEK [also known as Dsor] (Tsuda et al., 1993), DmErk [also known as rolled] (Biggs III and Zipursky, 1992), and corkscrew (Perkins et al., 1992).

The maternal terminal genes function to activate the expression of the zygotic genes, tailless (Strecker et al., 1986, 1988) and huckebein (Weigel et al., 1990). These zygotic genes encode putative transcription factors that are expressed in limited regions of embryonic poles. They are required for the subsequent development of the terminal structures (Pignoni et al., 1990; Brönner and Jäckle, 1991). Loss-of-function (l-o-f) of the maternal terminal gene products produced by the female
germline results in the deletion of anterior and posterior embryonic terminal structures in the embryo. Thus, in the absence of these gene products the anterior head skeleton is truncated and all structures posterior to the seventh abdominal segment are missing, including the Filzkörper. An exception to this is corkscrew. In the absence of the maternal corkscrew gene product, only the posterior endodermal derivatives are deleted and corkscrew embryos develop Filzkörper (Perkins et al., 1992).

A majority of the maternal terminal genes have been cloned and characterized. These gene products participate in a signal transduction pathway that is activated by the receptor tyrosine kinase torso (Casanova and Struhl, 1989; Sprenger et al., 1989), and the signal is mediated through the following cytoplasmic gene products: Drk encodes a SH3-SH2-SH3 protein that couples the receptor tyrosine kinase to Sos (Olivier et al., 1993; Simon et al., 1993); Sos encodes a guanine nucleotide exchange factor for Ras 1 (Buday and Downward, 1993); Ras 1 is homologous to mammalian Ki/Ha ras (p21ras) (Lu et al., 1993), which recruits Raf-1 kinase to the plasma membrane (Leevers et al., 1994; Stokoe et al., 1994); D-raf gene encodes a serine/threonine kinase that is homologous to the mammalian raf-1 proto-oncogene product (Mark et al., 1987; Nishida et al., 1988); Dsor encodes a putative protein kinase similar to the mitogen-activated protein (MAP) kinase activator (Tsuda et al., 1993); DmErfc encodes a functional homolog of a rat extracellular signal-regulated kinase (ERK), also known as the mitogen-activated protein (MAP) kinase (Biggs III and Zipursky, 1992). Finally, corkscrew encodes a putative non-receptor protein tyrosine phosphatase homologous to the mammalian PTP1C protein. This protein presumably acts to regulate the level of the torso signal (Perkins et al., 1992). A model for the Torso-mediated signal transduction is shown in Figure 1.

Structure and Function of D-raf

D-raf gene has been cloned (Nishida et al., 1988) and encodes the three conserved regions (CR1, CR2, and CR3) which are a common characteristic of the raf protein kinase family. The function of D-raf in the terminal class signal transduction pathway is similar to that of Raf-1 during the proliferation of mammalian cells. Raf-1 functions as a signal transducer for extracellular growth
Figure 1. Model for Torso signal transduction pathway.
factors and acts downstream of the receptor tyrosine kinase. Activation of the receptor results in the hyperphosphorylation of Raf-1 and stimulation of its serine/threonine kinase activity (Morrison et al., 1988). In addition, it has shown that activated Raf-1 protein can act as an intermediate to regulate the transcription of the c-fos and actin genes (Kaibuchi et al., 1989; Wasylyk et al., 1989; Jamal and Ziff, 1990). Recent biochemical studies have shown that D-raf has intrinsic serine/threonine kinase activity (Sprenger et al., 1993). Therefore, it is likely that D-raf, as a serine/threonine kinase, acts to transduce the terminal signal to activate the expression of tailless and huckebein.

At the amino acid level, there is 45% homology between D-raf and the mammalian Raf-1 with 65% identity in the serine/threonine kinase domain (Mark et al., 1987; Nishida et al., 1988). Primary amino acid analysis suggests that Raf-1 contains an N-terminal regulatory region and a carboxy-terminal catalytic region containing the kinase domain (CR3) (Bonner et al., 1986; Beck et al., 1987; Rapp et al., 1988). The mechanism by which the N-terminal region of Raf-1 regulates the molecule is not known, but it contains a cysteine rich region (CR1) and a serine/threonine rich domain (CR2) (Rapp et al., 1988). Recent research showed that Ras binds the CR1 domain of Raf-1 (Moodie et al., 1993; Van Aelst et al., 1993; Vojtek et al., 1993; Warne et al., 1993; Zhang et al., 1993) and recruits Raf-1 to the plasma membrane (Leevers et al., 1994; Stokoe et al., 1994). Removal of amino-terminal sequences results in the transforming activation of Raf-1, indicating the role of the amino-terminal region in the negative regulation of Raf-1 (Bonner et al., 1985). Also, the oncogenic v-raf protein is an amino-terminal truncation of the Raf-1 protein.

Regulation of Serine/Threonine Kinases

Protein kinases and phosphatases have been shown to play a critical role as mediators in signal transduction pathways. Protein kinases transfer the terminal phosphate group of ATP to a serine, threonine, or tyrosine residue of substrate proteins. Protein phosphatases dephosphorylate on a serine, threonine or tyrosine residue. Thus, reversible protein phosphorylation is a useful mechanism by which the regulation of cell fate choices can be made.
All protein kinases can be categorized into two general classes: serine/threonine-specific and tyrosine-specific. These kinases contain similar catalytic domains that have a highly conserved primary structure. The catalytic domain contains approximately 260 amino acid residues, corresponding to a molecular weight of 30 kDa (Hanks et al., 1988; Taylor et al., 1992). Eleven major conserved subdomains within the catalytic domain have been identified. It has been shown that these conserved subdomains function directly as components of the active site or indirectly by the formation of the active site. Interestingly, each class of kinases has certain specific short amino acid stretches. These sequences can be used to identify whether the kinase is tyrosine- or serine/threonine-specific (Hanks et al., 1988).

The biochemical studies in mammalian cells as well as in Drosophila have shown that the phosphorylation of Raf-1 and D-raf kinases occurs on serine and threonine residues by the activation of a receptor tyrosine kinase, suggesting that phosphorylation on serine and threonine residues is required for the signal transduction process (Morrison et al., 1988; Sprenger et al., 1993). By microinjecting mRNAs encoding various kinds of mutant Raf kinase, we address the role of D-raf in terminal class signal transduction pathway in Drosophila.

Dissertation Organization

This dissertation presents studies of D-raf in the terminal class signal transduction pathway. The three numbered chapters represent journal manuscripts. The chapters are followed by a general summary. The references cited in the general introduction are listed following the general summary.

Chapter 1 reports the development of a microinjection technique for Drosophila embryos. The work has been published in BioTechniques (Baek, K.-H., and Ambrosio, L. BioTechniques 17: 1024-1026, 1994).

Chapter 2 presents the requirement of D-raf association with the membrane and this is independent of activation of Torso receptor tyrosine kinase at the embryonic poles. My contribution included all the microinjection data and cuticular preparations. This work is the basis of a manuscript in preparation.
Chapter 3 deals with the temporal expression and dominant negative effects of D-raf. My contribution included all the microinjection data and cuticular preparations. This work is the basis of a manuscript in preparation.
CHAPTER 1: AN EFFICIENT METHOD FOR MICROINJECTION OF mRNA INTO DROSOPHILA EMBRYOS

A paper published in BioTechniques†

Kwang-Hyun Baek* and Linda Ambrosio

BACKGROUND

Microinjection of nucleic acids into embryos is commonly used to (i) determine gene identity (4), (ii) assay the effect of mutation upon gene function (5), (iii) identify genes that are expressed in stage- and/or tissue-specific patterns (2), and (iv) eliminate gene function (6). Here we describe an efficient protocol using both air and vacuum systems to maximize embryonic viability after microinjection. The key element of this procedure is the removal of cytoplasm that often leaks from *Drosophila* embryos after microinjection. We show that removal of this leaked cytoplasm leads to a significant increase in the percent of viable embryos. As a control experiment, nuclease-free distilled water was injected into embryos to determine their hatching rate after leaked cytoplasm was removed. We then tested this method after injection of *D-raf* mRNA into embryos lacking maternal D-raf protein. In both experiments a 40% increase in the number of embryos that completed development was observed.

Injection Procedure

Embryos were collected on molasses agar plates for 30 min and washed into a small basket. For basket construction, the bottom of a plastic scintillation vial was removed and a hole drilled into its cap, which was used to secure a 3 cm² 10-μm-mesh nylon screen (Spectrum Medical Industries, Houston, TX, USA). Chorions were removed by submerging the embryos into a bleach solution (50% Clorox®)


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for 3-5 min. The basket of embryos was rinsed thoroughly in a gentle stream of distilled water. Using forceps or a fine paint brush, 40-60 embryos were placed onto a patch of agar and then positioned to form a line with each embryo oriented in the same direction and not touching one another. The embryos were then transferred to the edge of a coverslip that had been streaked with a 400-μm-wide line of glue using the tip of a Pasteur pipet. Embryos were placed approximately 10-20 μm from the edge of the coverslip by gently touching them to the dried strip of glue. The glue was prepared by extracting pieces of brown packing tape with heptane overnight. For posterior injections, the posterior poles of the embryos were oriented towards the edge of the coverslip. To complete their preparation for injection, embryos were desiccated in a sealed jar containing Drierite™ for various times depending upon their genotype. Typically, they were dried 8-12 min (Optimal desiccation is achieved when the embryonic surface indents slightly beneath the needle during microinjection). After desiccation, the embryos were covered with a thin layer of Halocarbon oil (Series 95; Halocarbon Products, North Augusta, SC, USA).

Microinjections were performed using air (30 psi) and vacuum (700 mmHg) lines provided in house. A glass capillary needle was connected to a needle holder attached at an angle of 8°-10° to a micromanipulator (Model M-152; Narishige, Tokyo, Japan) that was mounted on an inverted microscope stage (Model CK2; Olympus, Lake Success, NY, USA). The needle holder was joined by Tygon™ tubing to a 3-way valve that was connected to air and vacuum systems. A second 3-way valve was inserted between the air/vacuum line and the needle. This valve was left open and regulated by placing a finger over its opening (Figure 1). For injection, each embryo was pierced by moving the stage toward the glass needle. The tip of the needle was positioned within the embryo at 5%-10% egg length (posterior pole = 0%) by moving the stage away from it. A volume approximately equal to 2%-5% of the embryo's total volume was injected using air pressure, and the needle was quickly withdrawn (slow removal of the needle leads to an increase of embryos with leaked cytoplasm). Wrinkled embryos were avoided. After the completion of a set of microinjections, embryonic cytoplasm that had leaked was removed by vacuuming, using a second large diameter glass capillary attached to the house vacuum line. An additional round of removal was carried out when needed. For mRNA injections, the tip of the needle containing mRNAs was kept
under oil when not in use. Occasionally, this tip became clogged with vitelline membrane residue and was reopened by touching its apex to the edge of the coverslip containing the embryos. After microinjection, embryos were kept on the coverslip under oil in a 3% agar petri dish and incubated at 19°C and 80% humidity for 24-48 h. Prior to use, the agar in the petri dish was moderately dried to prevent the oil from spreading. Additional oil can be added to keep embryos submerged, but care must be taken so that the oil does not leak under the coverslip. Important factors that influence the viability of microinjected embryos are listed in Table 1.

Needles were pulled from capillaries (o.d. 1.0 mm, i.d. 0.5 mm; World Precision Instruments, Sarasota, FL, USA) using a Vertical Pipette Puller (Model 700C; David Kopf Instruments, Pasadena, CA, USA). Sharp tips (a diameter of less than 6 μm) were generated by touching the apex of the needle to the debris of broken coverslips that had been placed on a glass slide and partially embedded under double-sided tape. The glass needle was back-filled using a heat-pulled Pasteur pipet. A second glass capillary with a diameter greater than 40 μm was made for vacuuming leaked cytoplasm.

For in vitro transcription, the template plasmid (pGEM7 [Promega, Madison, WI, USA] + D-raf cDNA) was purified using the Plasmid Midi Kit from Qiagen (Chatsworth, CA, USA). The DNAs were linearized, extracted with phenol (pH 7.4), precipitated with 0.1 vol of 3 M sodium acetate and 1.5 vol of ethanol and then dissolved in diethylpyrocarbonate (DEPC)-treated distilled water. mRNAs were generated using the mMESSAGE mmACHINE™ SP6 RNA Polymerase Kit from Ambion (Austin, TX, USA), and then dissolved in nuclease-free distilled water at 1.0 μg/μl.

D-raf mRNA Rescue

We first determined the percent embryonic survival (as assayed by cuticle formation) after removal of leaked cytoplasm for wild-type embryos injected with nuclease-free distilled water. As shown in Table 2, when leaked cytoplasm was removed, embryonic viability increased from 34% to 74%. In this experiment approximately 70%-80% of the embryos leaked cytoplasm. For the dechorionated but uninjected control embryos, 87% viability was observed. In a second
experiment, after injection of \textit{in vitro} synthesized wild-type \textit{D-raf} mRNA into embryos that lack maternal \textit{D-raf} activity, the number of embryos that completed development was 40\% higher when leaked cytoplasm was removed, as shown in Table 3. Embryonic viability was increased from 59\% to 99\%. The percentage of embryonic viability for uninjected embryos was 97\% in this experiment. Maternal \textit{D-raf} gene product is required for the determination of embryonic anterior and posterior cell fates (1). Without maternal \textit{D-raf}, female embryos produce cuticles that lack head and tail structures. Phenotypic rescue of this posterior defect was observed after injection of wild-type \textit{D-raf} mRNA (unpublished results), but the percent rescue was not influenced by the presence (80\%) or removal (85\%) of leaked cytoplasm. However, since the percentage of embryonic survival was greater when leaked cytoplasm was removed, this procedure greatly increases the efficiency of the microinjection technique. Fewer embryos need to be injected to obtain significant experimental results. This protocol may prove useful for the efficient microinjection of nucleic acids into various organisms including \textit{Drosophila}, \textit{Xenopus}, \textit{Caenorhabditis}, and \textit{Tetrahymena}.

\textbf{ACKNOWLEDGMENTS}

We thank Dr. E. Henderson for Figure 1, Drs. E. Henderson and J. Girton for critical reading of the manuscript, Dr. F. Sprenger for help and advice concerning the procedure and Drs. J. Fabian and D. Morrison for the \textit{D-raf} cDNA construct. This work was supported by NSF Grant IBN-9206580. Address correspondence to K.-H. Baek, Department of Zoology and Genetics, Signal Transduction Training Group, 3278 Molecular Biology Building, Iowa State University, Ames, IA 50011, USA. Internet: baek@iastate.edu
REFERENCES


Figure 1. *Drosophila* microinjection set up using in-house air and vacuum lines. Injection was performed by turning the air pressure line on and blocking the second value opening with a finger. Leaked cytoplasm was removed by turning the vacuum line on and covering the opening with a Pasteur-pipet bulb.
Table 1. Important Factors for High Viability of Microinjected Embryos

1. Dehydrate properly
2. Minimize and/or remove leaked cytoplasm
3. Maintain a thin layer of Halocarbon oil over embryos
4. Maintain high humidity
Table 2. Wild-Type Embryos were Injected with Nuclease-Free Distilled Water and Allowed to Develop for 24 h

<table>
<thead>
<tr>
<th>Leaked Cytoplasm</th>
<th>Embryos Assayed</th>
<th>Embryos Developed</th>
<th>Embryonic Viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Present</td>
<td>173</td>
<td>59</td>
<td>34 %</td>
</tr>
<tr>
<td>Removed</td>
<td>136</td>
<td>101</td>
<td>74 %</td>
</tr>
<tr>
<td>Uninjected</td>
<td>208</td>
<td>181</td>
<td>87 %</td>
</tr>
</tbody>
</table>
Table 3. Embryos from Homozygous \( D-raf^{11-29} \) Female Germline Clones were Injected with \( D-raf \) mRNA and Allowed to Develop for 48h

<table>
<thead>
<tr>
<th>Leaked Cytoplasm</th>
<th>Embryos Assayed*</th>
<th>Embryos with Cuticles</th>
<th>Posterior Rescue</th>
<th>Embryonic Viability</th>
<th>% Rescue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Present</td>
<td>85</td>
<td>50</td>
<td>40</td>
<td>59 %</td>
<td>80 %</td>
</tr>
<tr>
<td>Removed</td>
<td>79</td>
<td>78</td>
<td>66</td>
<td>99 %</td>
<td>85 %</td>
</tr>
<tr>
<td>Uninjected</td>
<td>67</td>
<td>65</td>
<td>0</td>
<td>97 %</td>
<td>0 %</td>
</tr>
</tbody>
</table>

* Only female embryos can develop cuticles (see Reference 3).
CHAPTER 2: SPATIALLY DISTINCT REQUIREMENTS FOR CONSTITUTIVE D-RAF FUNCTION IN TORSO SIGNAL TRANSDUCTION

A paper prepared for submission to Genes & Development

Kwang-Hyun Baek\(^1\), John R. Fabian\(^2\), Frank Sprenger\(^3\), Deborah K. Morrison\(^2\), and Linda Ambrosio\(^1,4\)

ABSTRACT

The Raf family of serine/threonine kinases are essential components in many receptor tyrosine kinase (RTK) mediated signal transduction pathways. Here, we analyze the function of D-raf in the Torso (Tor) pathway required to specify cellular fates at the embryonic poles. Using mutant embryos lacking endogenous D-raf function, we show that D-raf's serine/threonine kinase activity is essential for its role in Tor signal transduction and that human Raf-1 will substitute for D-raf in this pathway. After Tor activation, D-raf becomes hyperphosphorylated. We identified two serine phosphorylation sites (S388 and S743) and demonstrate that S743 or its phosphorylation is essential for D-raf function. N-terminal truncation of D-raf, serine-to-alanine substitution at S388, or targeted membrane association result in constitutive, Tor-independent signal transduction by D-raf at the poles. Interestingly, membrane targeted D-raf could promote development of terminal structures in the embryonic center, while N-terminal deleted and serine substituted D-raf did not. Together, these results suggest that at the embryonic poles there is an activity that facilitates localization of D-raf to the membrane compartment and this activity is independent of Tor activation. Thus, it is likely that determination

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of cellular fates and signal transduction by D-raf at the poles rely upon at least two upstream events, Tor independent localization and Tor dependent activation.

INTRODUCTION

Polarity along the anterior-posterior axis of the Drosophila embryo is established by the activity of maternal gene products deposited into the egg during oogenesis. These activities direct specialized domains of zygotic gene expression important for the determination of cellular fates (Klinger 1990; Nüsslein-Volhard 1991; St. Johnston and Nüsslein-Volhard 1992). Spatial information at the termini, or embryonic poles, is transferred to nuclei through the activity of the Torso (Tor) signal transduction pathway (Lu et al. 1993). Localized activation of the Tor receptor tyrosine kinase (RTK) (Sprenger and Nüsslein-Volhard 1992) leads to expression at the embryonic poles of zygotic genes encoding the putative transcription factors, tailless (Pignoni et al. 1990) and huckebein (Weigel et al. 1990; Bröner and Jäckle 1991), that are required for development of the embryonic head and tail.

The Tor protein is evenly distributed along the embryonic membrane (Casanova and Struhl 1989). Spatially restricted activation of Tor relies upon factors encoded by the fs(1) Nasrat (Degelmann et al. 1986), fs(1) polehole (Perrimon et al. 1986), trunk (Schüpbach and Wieschaus 1986), and torso-like (Stevens et al. 1990; Savant-Bhonsale and Montell, 1993; Martin et al. 1994) genes. Since these products act upstream of Tor, they are likely to play key roles in the production, accessibility and spatial distribution of the Tor ligand. Once Tor is activated along the syncytial membrane at the embryonic poles, its signal is transduced to underlying nuclei by an evolutionarily conserved set of proteins (Perrimon, 1993) that include D-ras1 (Lu et al. 1993), and the protein kinases D-raf (Nishida et al. 1988; Ambrosio et al. 1989a), D-MEK (Tsuda et al. 1993; Hsu and Perrimon, 1994; Lu et al. 1994), and MAP kinase (Biggs III and Zipursky 1992; Biggs III et al. 1994; Brunner et al. 1994).

How the activity of D-Raf is regulated in Drosophila is not well understood at this time. At the amino acid level, there is 45% homology between D-raf and the human Raf-1 proto-oncogene with 65% identity in the serine/threonine kinase domain (Mark et al. 1987; Nishida et al. 1988). In mammalian cells, the kinase
activity of Raf-1 is regulated by multiple mechanisms. Activation of Raf-1 after stimulation of cells with growth factors is accompanied by association of Raf-1 with Ras (Moodie et al. 1993; Van Aelst et al. 1993; Vojtek et al. 1993; Warne et al. 1993; Zhang et al. 1993), the translocation of Raf-1 to the plasma membrane (Leevers et al. 1994; Stokoe et al. 1994), and hyperphosphorylation of Raf-1 (Morrison et al. 1988; Morrison et al. 1989). In addition, since Raf-1 associates with other proteins including hsp 90, p50 (Stancato et al. 1993; Wartmann and Davis 1994), Fyn, Src (Williams et al. 1992; Cleghon and Morrison 1994), and 14-3-3 (Fartl et al. 1994; Freed et al. 1994; Fu et al. 1994; Irie et al. 1994), it is likely that Raf-1 exists as a member of a multi-protein complex(es) (Morrison 1994). It has also been demonstrated that Ras, Raf-1 and MEK can form a ternary complex (Huang et al. 1993; Moodie et al. 1993; Van Aelst et al. 1993; Jelinek et al. 1994).

We have examined the Tor pathway to learn about the regulation of D-raf and its signal transduction using microinjection of D-raf mRNAs into Drosophila embryos. As a single large cell, the Drosophila embryo serves as an in vivo system in which to assay the activity and biological function of various D-raf proteins. By injecting into the ends or middle of embryos, modified forms of D-raf can be expressed at the poles where the Tor pathway is active or in the center where it is inactive. In the absence of D-raf-mediated Tor signal transduction the embryonic head and tail fail to develop. Rescue of this defect by the microinjection of D-raf mRNA is used to assess whether propagation of the Tor signal has been achieved by D-raf. These effects were studied without interference from endogenous D-raf by preparing mutant embryos lacking maternal D-raf protein. We tested wild-type and modified D-raf proteins with serine substitutions that alter sites of phosphorylation, N-terminal truncations, and membrane associated D-raf fusion proteins, as well as human Raf-1 to characterize their activity in the Tor signaling pathway.

We find that D-raf's serine/threonine kinase activity is essential for rescue of the terminal defects in embryos lacking maternal D-raf. We also show that human Raf-1 can interact at low efficiency with Drosophila proteins to promote Tor signal transduction in embryos lacking maternal D-raf protein. In addition, we identified two sites of D-raf phosphorylation and show that one, serine S743, is essential for D-raf function in the Tor pathway. Tor independent, constitutive activity of D-raf at the embryonic poles is achieved by alanine substitution at S388, by N-terminal
truncation, and by targeting D-raf to the membrane. However, within the embryonic center only the membrane targeted forms of D-raf showed function. We propose that at the embryonic poles a Tor independent activity normally facilitates D-raf membrane association. Once localized to the membrane, D-raf interacts with its substrate(s) to propagate a functional signal.

**MATERIALS AND METHODS**

*Drosophila Strains*

In this study wild-type Oregon R; *D-raf*^11-29* (Ambrosio et al. 1989b; Melnick et al. 1993); *trunk^2*, *trunk^3* (Schüpbach and Wieschaus 1986; Lindsley and Zimm 1992); *D-MEK^LH110* (Lu et al. 1994) alleles were used. To generate germline clones of *D-raf*^11-29* and *D-MEK^LH110* the 'FLP-DFS' technique was utilized (Chou and Perrimon 1992).

*Basic Drosophila Techniques and Microinjections*

Flies were raised on *Drosophila* media at 25°C using standard conditions (Roberts 1986; Wieschaus and Nüsslein-Volhard 1986; Ashburner 1989). Embryos were collected on molasses agar plates and microinjected with *in vitro* synthesized mRNA using the procedure of Baek and Ambrosio (1994). Embryos at stages NC 9-12 were used for injection, and staging was according to Foe and Alberts (1983) and Campos-Ortega and Hartenstein (1985). After 48 hours at 19°C embryos were devitellinized with a sharp tungsten needle and Halocarbon oil (series 95, Halocarbon Products Corporation) was removed from each embryo with heptane. These cuticular preparations of embryos were embedded in a (1:1) mixture of Hoyer's: lactic acid according to van der Meer (1977) and photographed with a Zeiss Axioscope microscope using dark field illumination or Nomarski optics.

For *in vitro* translation of *in vitro* synthesized mRNA, mRNAs were translated using a rabbit reticulocyte lysate kit (Amersham). Each reaction contained 10 µl of reticulocyte lysate, 2 µl of 1 M potassium acetate, and 1 µg of *in vitro* synthesized
mRNA. Samples were incubated for 1 hr at 37°C and analyzed by SDS-polyacrylamide gel electrophoresis.

The D-raf gene is located on the X-chromosome and its product is required at multiple stages of development. Without injection, embryos that lack maternal D-raf protein show two phenotypic classes (Ambrosio et al. 1989b). Male embryos that lack maternal and paternal D-raf protein show little cuticle differentiation, and are not considered in this analysis. Female embryos lack maternal D-raf protein but inherit a paternal X-chromosome with a wild-type copy of the D-raf gene. These female embryos are defective only for the Tor signaling pathway. In this analysis, we address the question of posterior rescue for these female embryos. Thus, since the D-raf gene product acts in several different developmental pathways only its maternal role in the Tor pathway has been addressed here.

Plasmid Constructions

D-raf and Raf-1 Constructs

Wild-type D-raf plasmid is as described by Sprenger et al. (1993); Raf-1 as in Fabian et al. (1993); and Raf-1Δ305 as in Samuels et al. (1993).

Site-directed Mutagenesis

The plasmid pGEM7GLO was generated by inserting a 2.7 kb Hind III-Eco RI fragment from pSP64GLO, which contained the entire coding sequence of the D-raf gene (residues 1-782) downstream of a 56 bp 5'-untranslated leader sequence from the Xenopus β-globin gene, into the corresponding sites of plasmid pGEM7Z(-) (Promega). Single-stranded plasmid used for mutagenesis was produced from the plasmid pGEM7GLO upon infecting host cells with the helper phage M13K07 (Vieira and Messing 1987). Oligonucleotides were synthesized to generate the mutations K497M (5'-CCCGTGCCCGTAATGACACTCAACGTG-3'), S388A (5'-CAAGACGATCGATCCAATGCCGCGCCAAATGTG-3'), and S743A (5'-CATCGCAGTGCCGCTGAACCAAACTTG-3'). Mutants were generated by the method of Kunkel et al. (1987), as described by the vendor (for Biorad Mutagenesis Kit, Richmond, CA), and each mutation was confirmed by sequencing using a custom primer and Sequenase Version 2.0 DNA Sequencing Kit (United States Biochemical, Cleveland, OH).
Deletion Mutants

The deletion mutant D-raf$^{A315}$ was generated by removing a 0.9 kb Nco I fragment (encoding amino acids 1-315) from the construct pGEM7GLO and religating the plasmid. Deletion mutants D-raf$^{A370}$, D-raf$^{A445}$, and D-raf$^{A4021}$ were generated as described by Dickson et al. (1992). The deletion mutant D-raf$^{A445}$ was generated by PCR using the oligos 5'-CCGCGGCCATGGCCATGAGAGCAATAAATCTGC-3' and 5'-GGCCCGACGTCGCATGCTCCTCTAGACTCGAGG-3' as the 5'- and 3'- PCR primers, respectively. A 1.4 kb fragment was amplified (35 cycles of 94°C, 1.0 min., 56°C, 1.0 min., and 72°C, 2.0 min.), extracted with chloroform, ethanol precipitated and subsequently digested with Nco I and Eco RI. The PCR fragment was gel purified, extracted, and ligated into gel purified pGEM7GLO which had been digested with Nco I and Eco RI.

Expression of D-raf Proteins in Sf9 Cells

To obtain recombinant baculoviruses expressing the mutant D-raf proteins, DNA fragments containing wild-type and mutant D-raf coding sequences were isolated using the restriction enzymes Nco I and Eco RI and were inserted into the corresponding sites in the pAcC4 baculoviral transfer vector. Each of the recombinant transfer vectors were then co-transfected into Sf9 cells with wild-type AcNPV DNA, and recombinant baculoviruses isolated by plaque purification as described by Summers and Smith (1987).

Phosphopeptide Mapping

For peptide mapping, $^{32}$P labeled proteins isolated from Sf9 cells were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), eluted from the gel matrix, TCA precipitated, and subjected to enzymatic digestion with trypsin. Labeled polypeptides were separated on thin layer cellulose plates by electrophoresis at pH 1.9 for 27 min. at 1,000V, followed by ascending chromatography in a buffer containing n-butanol-pyridine-acetic acid-water in a ratio of 75:50:15:60.
Western Blot Analysis of Microinjected Embryos

Translation of injected mRNA in embryos was examined by Western blot analysis. After injection, the embryos were incubated at room temperature for 1 hr and then the Halocarbon oil was removed with heptane. To make extracts, the embryos were transferred into an Eppendorf tube and homogenized in lysis buffer containing 1% Nonidet P-40, 20 mM Tris (pH 8.0), 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, aprotinin (0.15 U/ml, 20 mM leupeptin per 1 ml (Sprenger et al. 1993). Insoluble material was removed by centrifugation at 4°C for 10 minutes at 10,000 x g.

D-raf protein was immunoprecipitated as described in Sprenger et al. (1993). Immunoprecipitates were resolved by 7.5% SDS-PAGE and transferred to 0.2 μm pore nitrocellulose membrane filters (Schleicher & Schuell) electrophoretically. These filters were blocked with 2% bovine serum albumin (Sigma) in Tris-buffered saline, pH 8.0 (TBS), for 1 hour, washed in TBST (TBS containing 0.2% Tween 20), and probed 2 hours with primary antibody diluted in TBST. The filters then washed in TBST, incubated with a horseradish peroxidase-coupled secondary antibody for 1 hour, and washed again in TBST. They were developed by using an enhanced chemiluminescence system (Amersham) and visualized on Kodak XAR-5 film.

RESULTS

Kinase Activity of D-raf is Essential for Its Role in Terminal Development

To determine whether the Tor signal transduction pathway could be reconstituted in embryos that lack maternal D-raf protein, we microinjected mRNA encoding either a wild-type D-raf (D-raf\textsuperscript{WT}) or kinase defective mutant D-raf (D-raf\textsuperscript{K497M}) protein (Sprenger et al. 1993). mRNA was injected at a concentration of approximately 1 μg/μl since this yielded levels of D-raf protein similar to that observed in wild-type embryos (Figure 1). After injection of D-raf\textsuperscript{WT} or D-raf\textsuperscript{K497M} mRNA, embryos were allowed to develop for 24-48 hours, and analyzed for the presence of terminal structures. Rescue was defined by scoring for Filzkörper. Since less than wild-type levels of D-raf activity are required for the formation of
this posterior structure (Melnick et al. 1993), this is a fairly relaxed (non-stringent) assay for Tor signal transduction.

Figure 2 shows cuticular preparations of a wild-type embryo (Figure 2A) and embryos that lack maternal D-raf protein from females with homozygous D-raf^{11-29} germlines (Figure 2B-F). In the absence of maternal D-raf, head and tail structures do not form (Figure 2B and 2E). After posterior injection of D-raf^{WT} mRNA, rescue of pattern including Filzkörper, tuft, and anal pads was observed (Figure 2C). Rescue of head structures was achieved when D-raf^{WT} mRNA was injected into the head region (Figure 2D and 2F). In contrast, injection of D-raf^{K497M} mRNA encoding the kinase defective form of D-raf into the anterior or posterior pole of the embryo rescued neither head nor tail defects. The results of this analysis are summarized in Table 1. To confirm the integrity of the mRNAs used in this assay D-raf mRNAs were translated in rabbit reticulocyte lysates and a 90 kDa protein, as expected for D-raf, was synthesized (data not shown). These results show that the kinase activity of D-raf is essential for its function in the Tor signaling pathway.

**Mammalian Raf-1 Proteins Can Substitute for D-raf in the Tor RTK Pathway**

We tested the ability of human Raf-1 to act in the Drosophila Tor signal transduction pathway since there is 45% amino acid homology between D-raf and Raf-1 (Mark et al. 1987; Nishida et al. 1988). In this experiment, in vitro synthesized Raf-1 mRNA (Fabian et al. 1993) was injected into the posterior of embryos lacking maternal D-raf protein. After injection, 70 embryos developed and of these 4 embryos showed terminal Filzkörper (5.7% rescue). Thus, Raf-1 can interact at low efficiency with Drosophila proteins in the Tor signal transduction pathway. We also tested whether a highly transforming N-terminal deletion of human Raf-1, Raf-1^{Δ305} (Samuels et al. 1993) could substitute for D-raf in embryos that lack maternal D-raf activity. In this case, 11% rescue of Filzkörper was achieved. These results indicate that there is conservation of structure amongst the components of this signaling cascade such that activator(s) and substrate(s) of D-raf are recognized by and can recognize human Raf-1 proteins.
Identification of D-raf Phosphorylation Sites

Phosphorylation may be a mechanism by which the activity of D-raf is regulated. Maternal D-raf protein becomes phosphorylated two hours after fertilization in wild-type embryos (Sprenger et al. 1993). At this time the Tor RTK has been activated and the intracellular signal generated for zygotic expression of tailless. Morrison et al. (1993) have identified the major phosphorylation sites of the Raf-1 kinase in mammalian and recombinant baculoviral infected Sf9 insect cells. Sequence comparison reveals that two of these phosphorylation sites (S259 and S621) and the residues surrounding these serines (RSXSXP) are conserved in all Raf family members, indicating that these sites of phosphorylation may be required for the enzymatic activity or regulation of the Raf kinases. These serine residues correspond to S388 and S743 of the D-raf protein.

To determine whether S388 and S743 are major in vivo sites of D-raf phosphorylation wild-type and mutant D-raf proteins with serine to alanine substitutions (S388A and S743A) were labeled with \[^{32}P\] orthophosphate in Sf9 cells co-infected with a baculovirus that expresses an activated form of the Tor receptor. The labeled D-raf proteins were immunoprecipitated from cell lysates, separated by electrophoresis on SDS-polyacrylamide gels, extracted, digested with trypsin, and the phosphopeptides were analyzed by two dimensional peptide mapping (Figure 3). For D-raf\(^{WT}\) five labeled peptides were observed (Figure 3A and D). For the altered D-raf proteins D-raf\(^{S388A}\) (Figure 3B) and D-raf\(^{S743A}\) (Figure 3C) phosphopeptides A and B, respectively, were absent (Figure 3D). This analysis indicates that serine residues 388 and 743 serve as D-raf phosphorylation sites in vivo. In addition, since the phosphopeptide map of kinase defective D-raf\(^{K497M}\) is identical to D-raf\(^{WT}\), it is unlikely that the phosphorylated peptides result from D-raf autophosphorylation (data not shown).

Signaling Activity of D-raf Proteins with Serine to Alanine Substitutions

To determine whether substitution of alanine at serine phosphorylation site 388 or 743 alters the ability of D-raf to participate in the Tor pathway, in vitro synthesized mRNAs encoding these and D-raf\(^{S388A/S743A}\) double mutant proteins were injected into the posterior of embryos lacking maternal D-raf protein. After
48 hours embryos were scored for the presence of Filzkörper and the results of these experiments are listed in Table 2. After injection with D-raf^S388A mRNA rescue of posterior pattern was observed at a level similar to that of D-raf^WT mRNA (78% as compared to 88%). However, D-raf^S743A and the double mutant D-raf^S388A/S743A mRNAs failed to promote the formation of terminal structures. Rescue of head structures was also observed after anterior injection of D-raf^S388A but not with D-raf^S743A or D-raf^S388A/S743A mRNAs (data not shown). Thus, the presence of a serine or phosphorylation at site 743 is essential for D-raf to propagate the Tor signal. In contrast, lack of a serine and/or phosphorylation at residue 388 does not interfere with D-raf's ability to act in the Tor pathway.

Activity of N-terminal Deleted and Membrane Associated Forms of D-raf

Amino terminal truncations of Raf-1 are transforming, the result of constitutive activation of Raf-1 kinase activity (Bonner et al. 1985; Rapp et al. 1988). Thus, removal of these residues relieves Raf-1 from its normal regulation (Heidecker et al. 1990). We tested whether D-raf proteins having N-terminal truncations would substitute for wild-type D-raf in the Tor pathway. For this analysis three deletions of D-raf were tested, D-raf^Δ315, D-raf^Δ370, and D-raf^Δ445 (Figure 4). D-raf^Δ315 lacks CR1 which contains the putative Ras-1 binding domain and a cysteine-rich region. D-raf^Δ370 lacks CR1 and part of CR2, but retains the rest of CR2 and the serine/threonine kinase domain (CR3). D-raf^Δ445 encodes only CR3. As shown in Table 3, when these deleted forms of D-raf mRNAs were injected into the posterior of embryos that lack maternal D-raf protein, greater than 50% rescue of Filzkörper was achieved. These results indicate that N-terminal truncated D-raf molecules can recognize and interact with their substrate(s) and transmit the Tor signal in Drosophila embryos.

It has been recently shown that targeting Raf-1 to the plasma membrane results in a Ras independent, active form of the Raf-1 kinase (Leevers et al. 1994; Stokoe et al. 1994). Therefore, we also tested two deletion mutations, D-raftor and D-raftor^Δ4021, which encode the CR2 and CR3 sequences equivalent to D-raf^Δ370 fused to the signal sequence, extracellular and transmembrane domains of the Tor protein. For D-raftor^Δ4021 the extracellular domain of Tor has a substitution of cysteine for tyrosine at position 327 that renders the protein constitutively active.
(Sprenger and Nüsslein-Volhard 1992). Since similar results were achieved for both forms of the chimeric Tor : D-raf protein, only the data for D-raf^{tor4021} are presented here. Both fusion proteins have previously been shown to induce R7 development in the Sevenless/D-ras1 signal transduction pathway in the absence of Sevenless RTK activity (Dickson et al. 1992). As shown in Table 3, 92% rescue of posterior Filzkörper was observed when D-raf^{tor4021} mRNA was injected into the posterior of embryos that lack maternal D-raf protein. Interestingly, a second effect of D-raf^{tor4021}, suppression of abdominal segmentation (Figure 5A-B) was observed for some of these embryos.

**Signal Transduction after Central Injection of Wild-Type and Modified D-raf mRNAs**

Sprenger and Nüsslein-Volhard (1992) showed that activation of the Tor pathway in the middle of wild-type embryos resulted in formation of terminal structures in the embryonic center and suppression of the normal segmented pattern. We tested for a similar effect by injecting wild-type and modified forms of D-raf mRNAs into the middle of embryos that lack maternal D-raf protein. As shown in Table 4, when embryos were injected in the center with D-raf^{WT} mRNA, rescue of both anterior and posterior defects was observed. Thus, D-raf was distributed to both ends of the embryo and participated in Tor-mediated signal transduction at the poles. This result is likely due to diffusion of the centrally injected D-raf mRNA and/or protein to the embryonic poles. Similar results were achieved after central injection of mRNAs encoding the D-raf^{S388A} and D-raf^{A445} proteins (Table 4; Figure 5F). However, when D-raf^{tor4021} mRNA was injected into the middle of embryos lacking maternal D-raf protein, suppression of abdominal segmentation as well as rescue of terminal structures was observed (Table 4). Furthermore, as shown in Figure 5C-E, when wild-type embryos were injected in the middle with D-raf^{tor4021} mRNA secondary Filzkörper developed centrally and abdominal segmentation was suppressed. Thus, membrane targeted D-raf promotes terminal development at the poles and in the middle of the *Drosophila* embryo.
Modified Forms of D-raf Act Independent of Tor Activation

To test whether D-rafS388A, D-rafΔ445, or D-rafΔtor4024 act with constitutive, Tor independent activity, each of these modified D-raf mRNAs were injected into embryos lacking trunk activity. Based upon genetic epistasis experiments trunk is positioned upstream of Tor in the signaling pathway and may encode the Tor ligand, or play a role in its accessibility. In these embryos little tyrosine phosphorylation of Tor is observed while high levels are detected for the wild-type activated Tor receptor (Sprenger et al. 1993). Table 5 lists the results of these experiments. After posterior injection into trunk embryos, rescue of Filzkörper was achieved for all mutant D-raf mRNAs tested. Thus, these modified forms of D-raf show constitutive activity at the poles, and in the absence of Tor activation transmit the terminal signal.

In addition, these modified D-raf mRNAs were tested in embryos that lack D-MEK activity. D-MEK has been shown to act downstream of D-raf in the Tor signaling pathway (Tsuda et al. 1993; Lu et al. 1994). Posterior structures were not produced after expression of the modified D-raf proteins in embryos lacking D-MEK, indicating that constitutive activation of D-raf did not result in utilization of aberrant signaling pathways (data not shown).

DISCUSSION

The Tor pathway is activated when the Drosophila embryo is a syncytium. In this single large cell the subcellular localization of maternal gene products has been detected using genetic approaches and also by direct visualization. At the anterior and posterior poles high concentrations of maternal bicoid and nanos mRNA accumulate, respectively (Nüsslein-Volhard 1991). Both protein products form gradients that direct determination of cell type and establish the anterioposterior embryonic axis. As a large single cell, the Drosophila embryo is easily amenable to phenotypic rescue by microinjection. Since mutations in various gene products of the Tor pathway have been characterized they serve as good backgrounds in which to add wild-type or to assay the activity of altered signaling components. Thus, we have used the Tor signal transduction pathway to test the function of modified D-raf proteins in an in vivo developmental context. D-raf mutations were
generated to address the mechanism(s) that regulates its activity or facilitates access to its substrate(s). This system offers a rapid approach for structure/function analysis of the Raf family of serine/threonine kinases as well as other molecules that participate in the evolutionarily conserved signaling module utilized by receptor tyrosine kinases (Perrimon 1993).

**Serine/Threonine Kinase Activity of D-raf is Essential for Tor Signal Transduction**

It has been previously shown that D-raf autophosphorylates and acts as a serine/threonine kinase *in vitro* when incubated with Mn$^{2+}$ and $[^{32}P]$ATP (Sprenger et al. 1993). In addition, Melnick et al. (1993) showed that two mutations of D-raf that gave a null phenotype in the Tor pathway were associated with single amino acid substitutions within the conserved region of D-raf's kinase domain. These two findings strongly indicate that D-raf functions as a serine/threonine kinase in *Drosophila*, and that this activity is required for Tor signal transduction. However, a kinase dependent as well as an independent requirement, related to subcellular localization and/or participation in a multiprotein complex, has been shown for the *Drosophila* Abelson tyrosine kinase (Henkemeyer et al. 1988; Henkemeyer et al. 1990). Thus, here we show directly that a point mutation that inactivates the D-raf serine/threonine kinase *in vitro* (Sprenger et al. 1993) also abrogates the ability of the protein to act in the Tor pathway. Thus, D-raf's kinase activity is essential for its function in Tor signal transduction.

**Conservation of Structure and Function of Signal Transduction Molecules**

We tested whether human Raf-1 could participate and showed that it can interact at low efficiency with *Drosophila* proteins in the Tor signal transduction pathway. In addition, the highly transforming N-terminal deletion form of Raf-1, Raf-1$^{Δ305}$ (Samuels et al. 1993), could substitute for D-raf in embryos that lack maternal D-raf activity. Overall, these results show that there is structural conservation between the components of this signaling cascade and that activator(s) and substrate(s) of D-raf are recognized by and can recognize the human Raf-1 proteins. Thus, it is likely that we can use the genetics of *Drosophila*
to gain a better understanding of the mechanisms utilized for signal transduction as it pertains to the control of growth and development in higher organisms.

**Phosphorylation as a Mechanism for D-raf Regulation**

Protein kinases and phosphatases play a critical role as mediators in signal transduction pathways. Reversible protein phosphorylation is a good mechanism by which to regulate developmental choices. Two phosphorylation sites have been mapped for the D-raf kinase, serine residue S388 and S743. These sites are phosphorylated when D-raf protein is isolated from Sf9 insect cells co-infected with activated Tor RTK and D-raf. These two residues and surrounding residues (RSXSXP) are conserved among members of the Raf family of protein kinases and correspond to serine 259 and 621 of the Raf-1 protein (Morrison et al. 1993). Since phosphorylation occurs at these sites for the kinase defective D-raf\textsuperscript{K497M} protein, these events are not due to autophosphorylation. Rather phosphorylation at these serine residues appears to be dependent upon another kinase(s) and thus, these sites are potentially important for the regulation of D-raf activity.

Substitution of an alanine at residue S743 rendered the D-raf protein inactive, while substitution at site S388 was permissive and resulted in constitutive activity independent of Tor activation. This result is consistent with that observed for Raf-1 when the homologous amino acid substitutions were tested. In in vitro kinase assays \textit{Raf-1}S\textsuperscript{621}A had no activity while \textit{Raf-1}S\textsuperscript{259}A had a two fold increase in kinase activity when compared to wild-type Raf-1 (Morrison et al. 1993). Thus, it appears that there is functional conservation of these two phosphorylation sites indicating that the mechanisms for modulating Raf activity are evolutionarily conserved. Phosphorylation could serve to alter the conformation of the Raf protein, its enzymatic activity, its interactions with other molecules, or a combination of these modifications.

**Regulation of N-terminal Deletion Mutants of D-raf**

The Tor receptor upon binding ligand likely undergoes dimerization, autophosphorylation (Sprenger et al. 1993), and recruitment of Drk and Sos to the membrane (Buday and Downward 1993; Olivier et al. 1993; Simon et al. 1993). In
As a result, it is likely that Sos promotes the exchange of GDP for GTP on D-ras1 which stimulates the interaction between D-ras1 and D-raf, facilitating the activation of D-raf which then activates D-MEK. The process is dynamic and likely requires movement and spatial rearrangement of multi-protein complexes. In embryos that lack maternal D-raf protein, this series of events is halted by the absence of the D-raf protein. It is in this context that truncated versions of D-raf can act to transmit a signal. We tested three different N-terminal deletions each encoding the D-raf kinase domain that effectively participated in signal transduction. This result indicates that at least a fraction of the truncated D-raf molecules recognize, have access to, and can interact with the substrate(s) of the full length wild-type D-raf protein.

**D-raf Membrane Association and Terminal Development in the Embryonic Center**

Raf-1 targeted to the plasma membrane by N-terminal fusion with a Ras membrane localization signal shows constitutive activation in assays of MEK and MAP kinase activities (Leevers et al. 1994; Stokoe et al. 1994). Its constitutive activation was confirmed by demonstrating that membrane localized Raf-1 induces neurite outgrowth in PC12 cells and morphological transformation of NIH3T3 cells (Leevers et al. 1994). Thus, it appears that membrane association of Raf-1 is a key toward achieving activation of downstream signaling components in the Raf/MEK/MAP kinase cascade. When an N-terminal truncated D-raf is targeted to the embryonic membrane by fusion with the signal sequence, extracellular and transmembrane domains of Tor, this chimeric D-raf protein directs the formation of Filzkörper at the posterior pole. In addition, this mutant D-raf protein suppresses the formation of abdominal pattern and directs the formation of terminal structures in the center of the embryo. Thus, by anchoring D-raf to the membrane it acts constitutively in the embryonic center to activate downstream components of the Tor signaling pathway.

Since membrane localization appears to be a key in achieving biological function for the Raf family of serine/threonine kinases, we propose that D-raf must also be recruited to the membrane for functional activation of its downstream target(s). Thus, when N-terminal deleted or serine substituted forms of D-raf were tested for constitutive activity by mRNA injection into the embryonic center,
Modified Forms of D-raf Act Constitutively and Independent of Tor Activation at the Poles

Activation of Tor RTK is regulated by a diffusible ligand produced at the extracellular terminal regions of the *Drosophila* embryo (Sprenger and Nüsslein-Volhard 1992). Since the ligand is limited in amount, and normally bound by Tor at the poles, this results in a spatially restricted pattern of activated Tor receptor. Based upon genetic epistasis experiments *trunk* is positioned upstream of Tor in the signaling pathway and may encode the Tor ligand, or play a role in its accessibility. In *trunk* mutant embryos little tyrosine phosphorylation of Tor is observed (Sprenger et al. 1993) correlating with the absence of head and tail structures later in development (Schüpbach and Wieschaus 1986). Thus, along the entire embryonic membrane for *trunk* embryos, and for the central membrane of
wild-type embryos, the Tor receptor is present, but is not activated. In these embryos, nuclei are directed to develop a non-terminal fate.

We find that when N-terminal deleted, serine substituted and membrane targeted mutant D-raf proteins are expressed at the posterior of trunk mutant embryos they act constitutively and direct the specification of terminal cell fates in the absence of Tor activation. Since these constitutive forms of D-raf do not rescue the Tor signaling defect in embryos that lack D-MEK activity, it is unlikely that propagation of these signals involve phosphorylation of aberrant substrates.

**A Model for Localization of D-raf to the Membrane**

In summary these findings indicate that association of D-raf with the membrane is essential for its biological function in Tor signal transduction. Here, we propose that in the Drosophila embryo there is an intracellular activity at the poles that facilitates association of the D-raf serine/threonine kinase with the membrane (Fig. 6A). This factor acts in the absence of Tor activation to recruit D-raf to the membrane (Fig. 6B). This factor may escort D-raf and/or facilitate its membrane association by providing a docking platform. It is possible that other components of this pathway also show membrane association at the termini. When Tor and/or D-ras1 are active within the central domain D-raf is recruited to the membrane by its interaction with activated D-ras1 (Fig. 6C). In this model activated D-raf phosphorylates and activates D-MEK which is a downstream target to propagate the Tor signal. Thus, in wild-type embryos two independent pathways are utilized at the poles for propagation of the Tor signal by D-raf. There is a Tor independent pathway that facilitates D-raf's association with the membrane and Tor dependent D-raf activation, e.g. phosphorylation by a Tor dependent kinase. This developmental strategy may have evolved to ensure efficient interaction between activated D-ras1 and D-raf by increasing the availability of D-raf molecules at the membrane. Thus, specification of terminal cell fates appears to rely on a system redundant for D-raf membrane localization. However, this mechanism may permit rapid transmission of extracellular signals during development in Drosophila.
ACKNOWLEDGMENTS

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REFERENCES


Table 1. Microinjection of $D$-raf mRNA into embryos that lack maternal $D$-raf protein.

<table>
<thead>
<tr>
<th>Region (egg length)</th>
<th>$D$-raf mRNA</th>
<th>Number of injected embryos</th>
<th>Number of embryos with cuticles*</th>
<th>Rescue of Filzkörper or medial tooth</th>
<th>Percent rescue (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Posterior wild-type (90% - 100%)</td>
<td>K497M</td>
<td>120</td>
<td>57</td>
<td>50</td>
<td>88 %</td>
</tr>
<tr>
<td>Anterior wild-type (0% - 10%)</td>
<td>K497M</td>
<td>137</td>
<td>75</td>
<td>65</td>
<td>87 %</td>
</tr>
</tbody>
</table>

* Male embryos from $D$-raf mutant germlines do not develop cuticles (Materials and Methods).
Table 2. Microinjection of serine to alanine substituted forms of D-raf mRNA into the posterior region of embryos lacking maternal D-raf protein.

<table>
<thead>
<tr>
<th>D-raf mRNA</th>
<th>Number of injected embryos</th>
<th>Number of embryos with cuticles*</th>
<th>Rescue of Filzkörper</th>
<th>Percent rescue (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S388A</td>
<td>143</td>
<td>80</td>
<td>62</td>
<td>78 %</td>
</tr>
<tr>
<td>S743A</td>
<td>196</td>
<td>92</td>
<td>0</td>
<td>0 %</td>
</tr>
<tr>
<td>S388A/S743A</td>
<td>151</td>
<td>83</td>
<td>0</td>
<td>0 %</td>
</tr>
</tbody>
</table>

* Male embryos from D-raf mutant germlines do not develop cuticles (Materials and Methods).
Table 3. Microinjection of mRNAs encoding N-terminal deleted and a membrane targeted D-raf into the posterior region of embryos lacking maternal D-raf protein.

<table>
<thead>
<tr>
<th>D-raf mRNA</th>
<th>Number of injected embryos</th>
<th>Number of embryos with cuticles*</th>
<th>Rescue of Filzkörper</th>
<th>Percent rescue (%)</th>
<th>Suppression of abdominal segmentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-rafA315</td>
<td>153</td>
<td>86</td>
<td>49</td>
<td>57 %</td>
<td>No</td>
</tr>
<tr>
<td>D-rafA370</td>
<td>120</td>
<td>68</td>
<td>37</td>
<td>54 %</td>
<td>No</td>
</tr>
<tr>
<td>D-rafA445</td>
<td>160</td>
<td>95</td>
<td>90</td>
<td>95 %</td>
<td>No</td>
</tr>
<tr>
<td>D-raford4021</td>
<td>85</td>
<td>51</td>
<td>47</td>
<td>92 %</td>
<td>Yes</td>
</tr>
</tbody>
</table>

* Male embryos from D-raf mutant germlines do not develop cuticles (Materials and Methods).
Table 4. Microinjection of mRNAs encoding wild type, a serine substituted, an N-terminal deleted, and a membrane targeted D-raf protein into the middle region of embryos lacking maternal D-raf protein.

<table>
<thead>
<tr>
<th>$D$-raf mRNA</th>
<th>Number of injected embryos</th>
<th>Number of embryos with cuticles*</th>
<th>Rescue of Filzkörper or medial tooth</th>
<th>Percent rescue (%)</th>
<th>Suppression of abdominal segmentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-raf$^{WT}$</td>
<td>136</td>
<td>68</td>
<td>49</td>
<td>72 %</td>
<td>No</td>
</tr>
<tr>
<td>D-raf$^{S388A}$</td>
<td>88</td>
<td>39</td>
<td>33</td>
<td>85 %</td>
<td>No</td>
</tr>
<tr>
<td>D-raf$^{A445}$</td>
<td>130</td>
<td>53</td>
<td>42</td>
<td>79 %</td>
<td>No</td>
</tr>
<tr>
<td>D-raf$^{tor4021}$</td>
<td>78</td>
<td>40</td>
<td>38</td>
<td>95 %</td>
<td>Yes</td>
</tr>
</tbody>
</table>

* Male embryos from D-raf mutant germlines do not develop cuticles (Materials and Methods).
Table 5. Microinjection of mRNAs encoding a serine substituted, an N-terminal deleted, and a membrane targeted D-raf into the posterior region of *trunk* mutant embryos.

<table>
<thead>
<tr>
<th>D-raf mRNA</th>
<th>Number of injected embryos</th>
<th>Number of embryos with cuticles</th>
<th>Rescue of Filzkörper</th>
<th>Percent rescue (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-rafS388A</td>
<td>271</td>
<td>115</td>
<td>32</td>
<td>28 %</td>
</tr>
<tr>
<td>D-rafA445</td>
<td>336</td>
<td>135</td>
<td>36</td>
<td>27 %</td>
</tr>
<tr>
<td>D-rafOZ1</td>
<td>346</td>
<td>207</td>
<td>48</td>
<td>23 %</td>
</tr>
</tbody>
</table>
Figure 1. Accumulation of D-raf protein resulting from in vivo translation of wild-type D-raf mRNA after microinjection into embryos that lack maternal D-raf protein were microinjected and after one hour of incubation at 22°C, processed and D-raf protein immunoprecipitated from embryonic extracts then resolved by SDS-PAGE (7.5 % polyacrylamide gel) and examined by immunoblotting using a D-raf specific antibody. (A) Accumulation of 90 kDa D-raf protein in wild-type embryos. (B) Lack of D-raf protein in embryos generated from homozygous D-raf11-29 germline females after injection of distilled water. (C-F) Accumulation of D-raf protein in embryos produced by D-raf11-29 germline females after injection of increased concentrations of wild-type D-raf mRNA. Embryos injected with 0.75 µg/µl - 1.0 µg/µl D-raf mRNA accumulate an essentially wild-type level of D-raf protein. Rescue of posterior pattern was observed for embryos injected with 0.50 µg/µl - 1.5 µg/µl D-raf mRNA.
Wild Type
(Oregon - R)

A  B  C  D  E  F

0.0 ug/ul
0.5 ug/ul
0.75 ug/ul
1.0 ug/ul
1.5 ug/ul
Figure 2. Induction of terminal structures in D-raf embryos that lack maternal D-raf protein after injection of wild-type D-raf mRNA. Cuticular preparations of embryos are shown after the completion of embryonic development. (A) A wild-type embryo from a wild-type mother with Filzkörper (arrow) and involuted head skeleton. (B) An embryo that lacks maternal D-raf protein, both head and tail structures are deleted. (C) An embryo from a homozygous D-raf+/flyll-29 germline female after injection of wild-type D-raf mRNA showing rescue of posterior terminal structures including Filzkörper (arrow). (D and F) Embryos from a homozygous D-raf+/flyll-29 germline female after injection of D-raf mRNA showing rescue of head skeleton. (E) Truncated head skeleton of an embryo lacking maternal D-raf protein. Abbreviations: (db) dorsal bridge; (vpp) ventral posterior process.
Figure 3. Two dimensional phosphopeptide maps of wild-type and D-raf proteins with serine to alanine substitutions. (A) D-raf^{WT} (B) D-raf^{S388A} (C) D-raf^{S743A} (D) Schematic representation of five D-raf phosphopeptides.
Phosphopeptides
Figure 4. Linear representations of the primary amino acid structure for wild-type and modified D-raf proteins. CR1 contains the putative Ras-1 binding domain and a cysteine zinc finger-like motif, the CR2 is rich in serines and threonines, and CR3 encodes the serine/threonine kinase domain. Listed are the three amino acid substitutions used in this study and their positions within D-raf. In addition three deletion mutations of D-raf, and a deleted, fusion D-raf protein targeted to the membrane by the signal sequence, extracellular and transmembrane domains of the Tor protein are depicted. Abbreviations: (EC) extracellular domain; (TM) transmembrane domain.
N-m-raf

Substitutions

D-raf

D-raf

D-raf

D-raf

D-raf
Figure 5. Induction of terminal structures in D-raf mutant and wild-type embryos after injection of D-raf$^{tor4021}$ or D-raf$^{A315}$ mRNA. Cuticular preparations of embryos are shown after the completion of embryonic development. (A and B) Embryos from a homozygous D-raf$^{11-29}$ germline female after injection with D-raf$^{tor4021}$ mRNA showing rescue of posterior terminal structures including Filzkörper with some suppression of abdominal segmentation (arrow). (C, D and E) Embryos from a wild-type mother after injection into their middle regions with D-raf$^{tor4021}$ mRNA showing suppression of abdominal segmentation and formation of extra Filzkörper material and Filzkörper (arrows). (F) Embryo from a homozygous D-raf$^{11-29}$ germline female after central injection of D-raf$^{A315}$ showing rescue of posterior terminal structures including Filzkörper (arrow) and wild-type pattern of abdominal segmentation. For these experiments, approximately 0.25-0.50 $\mu$g/\$\mu$l of D-raf$^{tor4021}$ mRNA was used for injection since higher concentrations resulted in abnormal embryonic development, and little cuticle was formed.
Figure 6. A model for induction of terminal development in *Drosophila*: At the poles of a wild-type *Drosophila* embryo there is an intracellular activity that facilitates association of the D-raf serine/threonine kinase with the membrane. Activation of Tor by ligand binding results in D-ras1 activation and promotes interaction between D-raf and D-ras1. D-raf is then activated in a Tor dependent manner and phosphorylates D-MEK its downstream target to propagate the Tor signal. Other components of this pathway may also show membrane association at the termini. A) A wild-type embryo with D-raf bound to its localizing factor at the posterior pole. This complex is absent along the membrane in the central domain and both Tor and D-ras1 remain inactive. (B) At the embryonic poles in trunk mutant embryos Tor is not activated but D-raf is recruited to the membrane by its localizing factor. This factor may escort D-raf to the membrane compartment and/or facilitate its membrane association by providing a docking platform. (C) When Tor and/or D-ras1 are active within the central domain D-raf is recruited to the membrane by its interaction with activated D-ras1. A torso mutant embryo that has constitutive (gain-of-function or g-o-f) is depicted here.
A. 

B. 

C. 

Y torso
YY activated torso
YY torso g-o-f
O ras-GDP
C ras-GTP
◆ D-raf inactive
◆ activated D-raf
□ D-raf docking protein
CHAPTER 3: TEMPORAL AND DOMINANT NEGATIVE EFFECTS OF D-RAF IN TORSO SIGNAL TRANSDUCTION

A paper prepared for submission to Development

Kwang-Hyun Baek, Kori Radke, and Linda Ambrosio*#

ABSTRACT

The maternal D-raf serine/threonine kinase acts downstream of Torso for specification of cell fates at the embryonic termini. We find that the optimal period at which activation and Torso signal transduction by D-raf occurs is between nuclear cycle 4 and 12. Also, we show that zygotic expression of the D-raf gene occurs between 2 and 3 hours after fertilization, or at nuclear cycle 13, 14 and blastoderm stages of development. Thus, little or no paternal D-raf protein acts in Tor signaling. We also show that maternal D-raf<sup>400B8</sup> and D-raf<sup>C2Z2</sup> proteins exhibit dominant negative effects and inhibit rescue of mutant embryos injected with D-raf<sup>WT</sup> mRNA. We tested three constitutive, Torso independent mutant forms of D-raf for suppression of these effects. Expression of membrane targeted D-raf directed formation of terminal structures in embryos containing either D-raf<sup>400B8</sup> or D-raf<sup>C2Z2</sup> proteins, and D-raf lacking serine at phosphorylation site S388 suppressed the effect of maternal D-raf<sup>400B8</sup> proteins, while suppression was not observed after expression of N-terminal truncated D-raf. Based upon these findings we propose that signaling by D-raf is involved in interaction with its substrate at a membrane-associated site. This site of action is blocked by the D-raf<sup>C2Z2</sup> protein, but not by D-raf<sup>400B8</sup>. Membrane targeted D-raf has an access to its substrate independent of this site, while D-raf<sup>S388A</sup> requires this site to interact with its substrate. Since N-terminal deleted D-raf does not suppress the effect of D-raf<sup>400B8</sup>, this indicates that N-terminal regulatory region permits D-raf access to its substrate in this background. We also characterized six other D-raf alleles for

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dominant effects, and showed the correlation of reduced levels of rescue after injection of wild-type D-raf mRNA with nonfunctional maternal D-raf proteins.

INTRODUCTION

In Drosophila, the Torso (Tor) signal transduction pathway represents a simple switch and ultimately provides nuclei that lie at the embryonic termini with information necessary to determine their fates (Casanova and Struhl, 1989; Sprenger et al., 1989). The D-raf serine/threonine kinase, acting downstream of the Tor receptor tyrosine kinase (RTK), is required at many developmental stages to propagate signals that regulate cellular proliferation, determination and differentiation. For pattern formation in the early embryo, maternal D-raf is responsible for specifying cell identities at the embryonic poles. The signal transduction cascade ultimately leads to the activation of zygotic tailless (Pignoni et al., 1990) and huckebein (Weigel et al., 1990; Bröchner and Jäckle, 1991) gene expression in nuclei at the termini. These transcription factors regulate expression of the structural genes needed for head and tail development. Activation of D-raf is also triggered by the sevenless (Dickson et al., 1992) and Drosophila EGF receptors (Brand and Perrimon, 1994), in larval and ovarian follicle cells respectively, for determination of cell identity. Thus, temporal as well as spatial control of D-raf activation is a mechanism by which cell choice is regulated during development.

Transmission of these cell determination signals from the cell membrane to the nucleus depends upon the function of an evolutionarily conserved signaling module consisting of Ras/Raf/MEK/MAPK homologs utilized in both invertebrate and vertebrate development (Egan and Weinberg, 1993; Perrimon, 1993; Dickson and Hafen, 1994). Following ligand binding, the receptor tyrosine kinase (RTK) dimerizes leading to transphosphorylation on tyrosine residues (van der Geer et al., 1994). These phosphotyrosines then serve as attachment sites for the adapter protein, Grb2 (Drk in Drosophila), that couples with the guanine nucleotide-releasing factor, Son of sevenless (Sos), forming the membrane-localized activating complex for Ras. Thus, the activation of the receptor tyrosine kinase (RTK) leads to the recruitment of the Grb2, Sos, the Ras GTPase, Raf, and
two other kinases MEK, a putative tyrosine/threonine kinase, and MAP kinase (Lu et al., 1993) to the membrane.

It has been demonstrated that the effects of nerve growth factor (NGF) and epidermal growth factor (EGF) on PC12 cell differentiation are different (Traverse et al., 1994). This is based upon differences in the extent and duration of MAP kinase activation in response to these two growth factors. NGF can induce prolonged activation and nuclear translocation of MAP kinases in PC12 phaeochromocytoma cells. In contrast, stimulation with EGF leads to a transient activation of MAP kinases without distinct nuclear translocation (Gotoh et al., 1990; Traverse et al., 1992). This suggests that differential activation of signal transduction pathways is the basis of the differential cellular response to different factors at different stages of development. We address in this report when the Tor signal transduction pathway is required for the development of embryo.

It is likely that to achieve maximal activation of Raf-1 a complicated series of independent and interdependent events occur. Raf-1 has an N-terminal regulatory region and a C-terminal catalytic region (Bonner et al., 1986; Beck et al., 1987; Rapp et al., 1988). The N-terminal contains a cysteine rich domain (CR1) and a serine/threonine rich domain (CR2). Recent research has demonstrated that Ras1 binds the CR1 domain of Raf-1 (Moodie et al., 1993; Van Aelst et al., 1993; Vojtek et al., 1993; Warne et al., 1993; Zhang et al., 1993) and recruits it to the membrane (Leevers et al., 1994; Stokoe et al., 1994). This association of Raf-1 with Ras1 may not affect the activity of Raf-1, but is used by the cell for localization of Raf-1 to the membrane. Williams et al. (1992) have found that cells co-transfected with Raf-1 and either v-Src or v-Ras showed limited Raf-1 autokinase activity. However, a synergistic effect was observed resulting in full activation of Raf-1 when cells were transfected with Raf-1, v-Ras, and v-Src. This indicates that the signal transduction pathway may not be linear, and that parallel pathways for Raf-1's activation likely exist.

Recently, Baek et al. (Submitted) have shown that the localization of D-raf to the membrane at the embryonic poles occurs independent of Tor activation. Three modifications of D-raf were used in this study: N-terminal truncation, alanine substitution at serine site S388, and addition of sequences that target D-raf to the membrane. Expression of these mutant D-raf proteins leads to constitutive Tor independent signal transduction at the poles. However, only membrane targeted
D-raf showed phenotypic activity in the embryonic center. Based upon these results, it was proposed that a Tor independent pathway facilitates D-raf association with the membrane at the poles. In the current model, activation of Tor results in the formation of D-ras/D-raf complexes. After the activation of the pathway, D-ras/D-raf complexes are formed to promote the efficient activation of D-raf in a Tor dependent manner. D-raf then acts to phosphorylate and activate D-MEK, which is a downstream target to propagate the Tor signal. Thus, for Tor signal transduction by D-raf, at least two inputs are required, association with the membrane to localize D-raf to its activator(s) and/or substrate(s) and Tor dependent D-raf activation.

Biochemical studies have shown that hsp 90 forms heteromeric complexes with a 50 kDa protein (p50) and other proteins (Whitelaw et al., 1991). Also, 300-500 kDa Raf complex was detected during gel filtration chromatography. It is likely that this complex contains two molecules of hsp 90, one (or more) molecule(s) of p50, and Raf protein kinase (Wartmann and Davis, 1994). It has been reported that in *Saccharomyces cerevisiae* the activation of a protein kinase cascade, transmitting the signal through Ste11 (a MEKK), Ste7 (a MEK), and Fus3/Kss1 (a MAPK), is required for mating. Genetic and biochemical studies have shown that STE5 binds to STE11, STE7, and either FUS3 or KSS1, simultaneously (Choi et al., 1994). Since it has also been reported that Ras and 14-3-3 protein bind Raf-1 (Moodie et al., 1993; Van Aelst et al., 1993; Vojtek et al., 1993; Warne et al., 1993; Zhang et al., 1993; Fantl et al., 1994; Freed et al., 1994; ; Fu et al., 1994; Irie et al., 1994) and Raf-1 binds MEK (Huang et al., 1993; Macdonald et al., 1993; Jelinek et al., 1994), it is possible that there is a multiprotein complex at the membrane to transduce the signal.

The Raf family of serine/threonine kinases have also been shown to participate in vertebrate development in *Xenopus* for progesterone-stimulated meiotic maturation of oocytes (Fabian et al., 1993) and FGF-induced formation of posterior embryonic mesoderm (MacNicol et al., 1993). For both of these signaling pathways, the requirement for Raf-1 function was identified by expression of a kinase defective Raf-1 protein that blocked induction of either oocyte maturation or formation of embryonic mesoderm. Although the mechanism by which the mutant Raf-1 exhibited a dominant negative effect and inhibited endogenous Raf-1 function was not determined, it was suggested that this may be due to sequestration of a positive regulator required for Raf-1 activity. In murine cells it
was shown that the activation of wild-type endogenous Raf-1 was blocked by expression of the amino terminal half of Raf-1 due to its interaction with a positive activator (Kolch et al., 1991; Bruder et al., 1992). We address here whether nonfunctional D-raf proteins can affect the signal transduction of D-raf proteins translated in vivo by microinjection.

In this study we characterize the temporal requirements for D-raf activity and show that between nuclear cycle 4 (NC 4) and 12 activation and signal transduction by D-raf in the Tor pathway is optimal. In addition, we show that zygotic expression of the D-raf gene begins at 2-3 hours after fertilization (NC 13 - blastoderm stages). Thus, this finding is in agreement with phenotypic analyses that indicate the paternal D-raf gene does not contribute to signaling in the Tor pathway (Perrimon et al., 1985; Ambrosio et al., 1989). Here we also test whether Tor signal transduction is influenced by pools of endogenous mutant D-raf protein. We find that in the presence of maternal D-raf$^{400B8}$ and D-raf$^{C2Z2}$ proteins rescue by injection of D-raf$^{WT}$ mRNA is inhibited. Three constitutively active forms of D-raf were tested for suppression of these effects. By targeting D-raf to the membrane, both dominant negative effects were suppressed and rescue at the posterior pole was achieved. However, D-raf$^{S388A}$ containing an alanine at serine phosphorylation site S388, suppressed the effect of maternal D-raf$^{400B8}$ but not D-raf$^{C2Z2}$ proteins. N-terminal truncated D-raf did not show rescue. Together, these results are consistent with the idea that in the Tor signaling pathway, maternal D-raf$^{C2Z2}$ occupies a site utilized for D-raf's interaction with its substrate(s). This defect is suppressed by targeting D-raf directly to the membrane. In the case of endogenous D-raf$^{400B8}$ this site of action becomes available to full length D-raf$^{S388A}$, but not N-terminal truncated D-raf. We also characterized six other D-raf alleles for dominant effects, and showed that there is a correlation of reduced levels of rescue with nonfunctional maternal D-raf$^{DF903}$ and D-raf$^{raf2}$ proteins.

**MATERIALS AND METHODS**

*Drosophila Strains*

In this study, wild-type Oregon R and D-raf mutant alleles utilized are as described in previous reports (Nishida et al., 1988; Lindsley and Zimm 1992;
To generate germline clones of \textit{D-raf} alleles, the 'FLP-DFS' technique was utilized (Chou and Perrimon, 1992).

\textit{Basic Drosophila Techniques and Microinjections}

Flies were raised on \textit{Drosophila} media at 25°C using standard conditions (Roberts, 1986; Wieschaus and Nüsslein-Volhard, 1986; Ashburner, 1989).

Embryos were collected on molasses agar plates and microinjected with \textit{in vitro} synthesized mRNA using the procedure of Baek and Ambrosio (1994). For microinjection, staging was determined according to Foe and Alberts (1983) and Campos-Ortega and Hartenstein (1985). After 48 hours at 19°C embryos were devitellinized with a sharp tungsten needle and Halocarbon oil (series 95, Halocarbon Products Corporation) was removed from each embryo with heptane. These cuticular preparations of embryos were embedded in a (1:1) mixture of Hoyer's: lactic acid according to van der Meer (1977) and photographed with a Zeiss Axioscope microscope using dark field illumination.

For \textit{in vitro} translation of \textit{in vitro} synthesized mRNA, mRNAs were translated using a rabbit reticulocyte lysate kit (Amersham). Each reaction contained 10 \(\mu\)l of reticulocyte lysate, 2 \(\mu\)l of 1 M potassium acetate, and 1 \(\mu\)g of \textit{in vitro} synthesized mRNA. Samples were incubated for 1 hr at 37°C and analyzed by SDS-polyacrylamide gel electrophoresis.

The \textit{D-raf} gene is located on the X-chromosome and this gene product is required at multiple stages of development. Without injection, embryos that lack maternal \textit{D-raf} protein show two different phenotypic classes (Ambrosio et al., 1989). Male embryos that lack maternal and paternal \textit{D-raf} protein show little cuticle differentiation, and are not considered in this analysis. Female embryos lack maternal \textit{D-raf} protein but inherit a paternal X-chromosome with a wild-type copy of the \textit{D-raf} gene. These female embryos are defective for the Tor signaling pathway. In this analysis, we address the question of posterior rescue for these female embryos. Thus, since the \textit{D-raf} gene product acts in several different developmental pathways only its maternal role in the Tor pathway has been addressed here.
Plasmid Constructions

Wild-type and modified D-raf plasmids are as described in Baek et al. (Submitted).

Western Blot Analysis of Embryos

Embryos were collected 0-2 and 2-4 hours after egg laying, dechorionated, transferred to an Eppendorf tube and homogenized in lysis buffer containing 1% Nonidet P-40, 20 mM Tris (pH 8.0), 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, aprotinin (0.15 U/ml, 20 mM leupeptin at a concentration of 1 embryo per 1 μl (Sprenger et al., 1993). Insoluble material was removed by centrifugation at 4°C for 10 minutes at 10,000 x g.

D-raf protein was immunoprecipitated as described in Sprenger et al., (1993). Immunoprecipitates were resolved by 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred to 0.2 μm pore nitrocellulose membrane filters (Schleicher & Schuell) electrophoretically. These filters were blocked with 2% bovine serum albumin (Sigma) in Tris-buffered saline, pH 8.0 (TBS), for 1 hour, washed in TBST (TBS containing 0.2% Tween 20), and probed 2 hours with primary antibody diluted in TBST. The filters then washed in TBST, incubated with a horseradish peroxidase-coupled secondary antibody for 1 hour, and washed again in TBST. They were developed by using an enhanced chemiluminescence system (Amersham) and visualized on Kodak XAR-5 film.

RESULTS

Age Dependent D-raf Mediated Rescue

Tor protein first appears at nuclear cycle 4 (NC 4) on the surface of the embryonic membrane (Casanova and Struhl, 1989) and zygotic tailless (tll) gene expression begins about 60 minutes later at NC 11 (Pignoni et al., 1990; 1992). It is during this interval that activation of Tor and D-raf is required to transduce the terminal signal from the membrane into nuclei at the embryonic poles. To determine when activation of D-raf is required to transmit the Tor signal, D-rafWT
mRNA at 1 μg/μl was injected into embryos from mothers with D-raf\textsuperscript{11-29} homozygous mutant germlines. These embryos lack maternal D-raf protein (Sprenger et al., 1993). When embryos were injected at cleavage stages (NC 4-8) or at the time of pole cell formation (NC 9-12), 83% and 75% of the embryos developed Filzkörper, respectively (Table 1). However, at later syncytial stages (NC 13-14A) the percent rescue was dramatically reduced (23%) and after the onset of cellularization none of the injected embryos developed Filzkörper. Sprenger and Nüsslein-Volhard (1992) observed a similar age dependent rescue of torso mutant embryos after the injection of torso mRNA. This experiment indicates that the optimal period of activation and signal transduction by D-raf in the Tor pathway is between NC 4 and 12.

Temporal Control of Zygotic D-raf Expression

Phenotypic analyses have shown that a paternally inherited wild-type D-raf gene does not contribute to Tor-mediated signal transduction (Perrimon et al., 1985; Ambrosio et al., 1989). We used the D-raf\textsuperscript{PB26} allele to characterize the accumulation of paternally encoded D-raf protein. This is a hypomorphic allele of D-raf, and sequence data suggests that this form of the gene encodes a 75 kDa D-raf protein (Melnick et al., 1993). Embryos from D-raf\textsuperscript{PB26} germlines were collected at 0-2 and 2-4 hours after egg laying, lysates were prepared and then D-raf\textsuperscript{PB26} proteins were detected by immunoblot analysis. These embryonic lysates were compared to those obtained from wild-type germlines. As shown in Figure 1A a truncated approximately 75 kDa D-raf protein was observed in embryos from D-raf\textsuperscript{PB26} germlines while for wild-type embryos a 90 kDa D-raf protein was observed (Figure 1A).

Next, males carrying D-raf\textsuperscript{PB26} on their X-chromosome and a duplication (Dp(1;Y)\textsuperscript{zw+303}) of the wild-type D-raf gene on their Y-chromosome (see Lindsley and Zimm, 1992) were mated to wild-type females. Then embryos were collected in one hour intervals and assayed for the presence of the paternal D-raf\textsuperscript{PB26} protein by immunoblot analysis. As shown in Figure 1B, the accumulation of D-raf\textsuperscript{PB26} proteins was observed at 2-3 hours after fertilization (NC 13 - blastoderm stages). Since optimal rescue of the Tor pathway by injection of D-raf mRNA was observed
until NC 12, this finding is in agreement with phenotypic analyses that indicate there is no paternal D-raf contribution to the Tor pathway.

**Signal Transduction in the Torso Pathway is Influenced by Maternal D-raf Pools**

To determine whether rescue of posterior pattern is influenced by endogenous pools of nonfunctional maternal D-raf protein, D-raf<sup>WT</sup> mRNA was injected into embryos from homozygous D-raf<sup>400BB</sup> and D-raf<sup>C2Z2</sup> germlines. Based upon sequence characterization by Melnick et al., (1993) nonfunctional maternal D-raf protein should accumulate in embryos with these genetic backgrounds. To confirm this prediction, embryos from D-raf<sup>400BB</sup> and D-raf<sup>C2Z2</sup> germlines were collected at 0-2 and 2-4 hours after egg laying, lysates were prepared and then D-raf mutant proteins were detected by immunoblot analysis. These embryonic lysates were compared to those obtained from wild-type and D-raf<sup>11-29</sup> germlines. As shown in Figure 2, a 90 kDa D-raf protein accumulates in wild-type and in embryos from D-raf<sup>400BB</sup> and D-raf<sup>C2Z2</sup> mutant germlines, but is missing from D-raf<sup>11-29</sup> derived embryos. As shown in Table 2, after injection of D-raf<sup>WT</sup> mRNA neither D-raf<sup>400BB</sup> nor D-raf<sup>C2Z2</sup> germline embryos developed Filzkörper, although 86% rescue was obtained for embryos that lack maternal D-raf protein (Table 2). Thus, nonfunctional maternal D-raf protein exhibits a dominant negative effect and inhibits rescue by D-raf<sup>WT</sup> mRNA injection.

Recently, we have shown that three modifications of D-raf result in constitutive Tor independent D-raf activity (Baek et al., Submitted). We tested whether these modified versions of D-raf would suppress the dominant negative effect of the maternal D-raf<sup>400BB</sup> and D-raf<sup>C2Z2</sup> proteins (Table 2). D-raf<sup>Tor4021</sup>, is a membrane targeted D-raf protein and contains the signal sequence, extracellular and transmembrane domains of the Tor protein fused to a deleted D-raf that is missing amino acids 1-370. For D-raf<sup>S388A</sup> serine phosphorylation site 388, which is conserved in all Raf family members (Morrison et al., 1993), is substituted with an alanine. D-raf<sup>A445</sup> is missing amino acids 1-445 and encodes only the C-terminal serine/threonine kinase domain (CR3) of D-raf.

The results of this analysis are listed in Table 2. The dominant negative effects of endogenous D-raf<sup>400BB</sup> and D-raf<sup>C2Z2</sup> proteins were suppressed and posterior rescue was achieved by injection of D-raf<sup>Tor4021</sup> mRNA. However, for injection
with D-raf$^{388A}$ mRNA, suppression of only the D-raf$^{400B8}$ effect was observed. Rescue was not achieved in either maternal background after injection of D-raf$^{A445}$ mRNA. Figure 3A shows the cuticular pattern of a wild-type embryo after completion of embryonic development. In contrast, a non-rescued D-raf$^{400B8}$ derived embryo is shown in Figure 3B after injection of D-raf$^{WT}$ mRNA. Both head and tail structures are missing. However, rescue of posterior pattern including Filzkörper was observed (Figure 3C) after injection of D-raf$^{388A}$ mRNA.

**Characterization of Other Maternal Effect Alleles of D-raf**

Since embryos from D-raf$^{400B8}$ and D-raf$^{C222}$ germlines were not rescued by injection of D-raf$^{WT}$ mRNA, six additional alleles of D-raf were characterized by D-raf$^{WT}$ mRNA injection (Table 3). At least 68% rescue was achieved for each of these mutant backgrounds with the exception of D-raf$^{raf2}$ and D-raf$^{DF903}$, showing 37% and 45% rescue, respectively. Rescue of posterior pattern was also observed in all cases after injection of D-raf$^{388A}$ and D-raf$^{A445}$ mRNAs. In general, the % rescue was slightly higher after injection of D-raf$^{388A}$ mRNA, as compared with either D-raf$^{WT}$ or D-raf$^{A445}$ mRNAs. Next, we characterized the accumulation of D-raf protein in embryos from these D-raf mutant germlines. As shown in Figure 4, a 90 kDa D-raf protein is observed in wild-type embryos, and in embryos from homozygous D-raf$^{raf2}$ and D-raf$^{DF903}$ germlines. Thus, there is a correlation between the presence of mutant maternal D-raf protein and the lower level of rescue by D-raf$^{WT}$ mRNA injection. In the case of D-raf$^{DC817}$, approximately 82 kDa truncated D-raf protein is observed, while all remaining D-raf maternal backgrounds including D-raf$^{EA75}$ (Figure 4D) lacked D-raf protein.

**DISCUSSION**

**Temporal and Spatial Expression of D-raf**

The establishment of temporally and spatially restricted patterns of gene expression is a mechanism utilized during development to achieve specificity of cell type. In the early *Drosophila* embryo the maternal D-raf gene product participates in a signal transduction pathway mediated by Tor RTK for
specification of cellular fates at the embryonic poles. Positive regulation of this pathway is required for expression of tailless and huckebein genes to direct formation of head and tail structures. D-raf is also utilized in other cell types at later stages of development to propagate signals generated by the sevenless RTK for determination of the R7 photoreceptor (Dickson et al., 1992) and the DER (Drosophila EGF Receptor) pathway for establishment of dorsal/ventral polarity in follicle cells of egg chambers (Brand and Perrimon, 1994). Thus, temporal regulation of D-raf's activity is related to its specific cellular function. We find that the optimal period of activation and signal transduction by D-raf in the Tor pathway is between NC 4 and 12. We also show that expression of paternally inherited D-raf begins between NC 13 and blastoderm stages of development and does not contribute to Tor signaling.

**Dominant Negative Effects of Endogenous Nonfunctional D-raf**

How D-Raf's activity is regulated in *Drosophila* development is not well understood at this time. However, based upon biochemical and distribution studies of the Raf family of serine/threonine kinases, localization, activation and substrate accessibility are important factors when considering the regulation of their biological function. Here, a genetic approach is utilized to investigate the mechanism(s) that regulate signal transduction by D-raf. Using mutations that block the pathway at specific points we hope to dissect the molecular steps required for propagation of the Tor signal by D-raf. In previous studies, the maternal defects associated with lack of D-raf protein were rescued by microinjection of wild-type D-raf mRNA (Baek and Ambrosio, 1994; Baek et al., Submitted).

To test whether rescue of posterior pattern is influenced by endogenous pools of nonfunctional maternal D-raf protein, D-rafWT mRNA was injected into embryos from homozygous D-raf400B and D-rafC222 germlines. Phenotypically, embryos from these two genetic backgrounds have the same terminal deletions as those from D-raf11-29 germline clones. Pattern elements posterior to abdominal segments 6 or 7 are deleted and anteriorly, a portion of the cephalopharyngeal skeleton, the labial sense organ, and the medial tooth are missing (Ambrosio et al., 1989). Based upon sequence characterization by Melnick et al. (1993), kinase
defective maternal D-raf proteins should accumulate in these embryos. Using Western blot analysis of lysates from these embryos, we show that a 90 kDa D-raf protein accumulate in each of these genetic backgrounds. Wild-type levels of D-raf protein were present for D-raf^{400B8} and D-raf^{C222} germline embryos. However, rescue was not observed after injection of D-raf^{NT} mRNA. Thus, both D-raf^{400B8} and D-raf^{C222} proteins showed dominant negative effects. Based upon the interpretation of similar effects of kinase defective Raf-1 in vertebrates (Kolch et al., 1991; Bruder et al., 1992; Fabian et al., 1993; MacNicol et al., 1993), it is likely that a factor required for wild-type D-raf activation is sequestered by the mutant D-raf^{400B8} and D-raf^{C222} proteins.

*Mutation of D-raf^{400B8} and D-raf^{C222}*

Then, how does each mutation in the D-raf^{400B8} and D-raf^{C222} proteins affect their activity? It has been predicted that these proteins are kinase defective based upon sequence characterization and the structure of cAMP-dependent protein kinase as a model (Melnick et al., 1993). In general, Mg\(^{2+}\)ATP and substrate binding take place within the cleft formed between the small and large lobes of a kinase (Taylor et al., 1992). The glycine rich nucleotide binding motif existing in all kinases lies within the small lobe and is responsible for association of Mg\(^{2+}\)ATP with Lys 72 and Glu 91 of the large domain. The amides of these glycines have an access to and a hydrogen bond with the non-transferable \(\beta\)-phosphates of ATP. In this way the glycine loop serves as a clamp that helps to lock the non-transferable \(\beta\)-phosphate into place, together with Lys 72 and Glu 91, and the hydrophobic pocket that binds the adenine ring provides a docking site for Mg\(^{2+}\)ATP. Once the nucleotide is bound, the glycine loop can clamp it firmly into its proper position (Taylor et al., 1992).

Using cAMP-dependent protein kinase as a model, we expect that these three glycines play an important role in D-raf nucleotide binding. The *D-raf^{400B8}* mutation results in the substitution of an arginine for the third glycine of the Gly-X-Gly-X-X-Gly nucleotide binding motif. The *D-raf^{C222}* mutation results in the substitution Glu\(^{516}\) to valine (Melnick et al., 1993). Glu\(^{516}\) is on the edge of an alpha helix in the small lobe of the kinase domain. It is analogous to Glu\(^{91}\) of cAMP-dependent protein kinase that together with Lys72 and Asp184 are
important for Mg\(^{2+}\)ATP binding and are in close proximity. It is possible that both mutant D-raf\(^{1400B8}\) and D-raf\(^{C2Z2}\) proteins interact with upstream factors. However, it remains unclear whether either will bind and/or release substrate(s). Based upon the results that catalytically inactive Raf-1 can associate with Ras, 14-3-3, and MEK (Finney et al., 1993; Freed et al., 1994; Hallenberg et al., 1994; Stokoe et al., 1994), we suggest that mutant D-raf proteins may form inactive protein complexes upon activation of Tor signaling pathway so that wild-type D-raf proteins translated from injected wild-type D-raf mRNA in these backgrounds cannot function.

Suppression of the Dominant Negative Effects

Next, we tested for suppression of the dominant negative effects of the maternal D-raf\(^{1400B8}\) and D-raf\(^{C2Z2}\) proteins by injection of mRNAs encoding constitutive forms of D-raf. Thus, for these mutant D-raf proteins, activation is no longer required but proper localization and substrate(s) accessibility must be considered to achieve the biological function. Both dominant effects were suppressed by the membrane targeted D-raf. However, a similar N-terminal deleted D-raf that lacks the membrane localization signal did not suppress these effects. In addition, we found that D-raf lacking serine at phosphorylation site S388 suppressed the dominant negative effect of D-raf\(^{1400B8}\) proteins but not D-raf\(^{C2Z2}\) proteins. D-raf\(^{S388A}\) is a full length D-raf and contains the entire N-terminal regulatory region including the putative D-rasl binding domain and a conserved cysteine motif (CR1). These portions of the molecule are absent in the truncated D-raf protein.

Since membrane targeted D-raf suppresses dominant negative effects, this indicates that the localization to the membrane allows D-raf accessibility to its substrate(s). Full length activated D-raf\(^{S388A}\) also has access to its substrate(s) in the D-raf\(^{1400B8}\) background, while truncated D-raf does not. Apparently, the N-terminal domain of D-raf is required for substrate access after the pathway has been activated, since the access to substrate by the N-terminal region is blocked. This result is in contrast to that shown in the absence of Tor activation for trunk mutant embryos that N-terminal deleted forms of D-raf have access to and can activate downstream target(s) at the posterior pole.
It is likely that proper localization, activation, and access to substrates are required for wild-type D-raf function. In embryos where Tor RTK is inactive, at the poles, all three constitutive forms of D-raf are likely localized and have access to their substrates. In embryos where Tor has been activated but which lack D-raf proteins, these three forms of D-raf can also transmit the signal, suggesting that they become properly localized and have access to their substrates. However, when a mutant form of D-raf is present, wild-type D-raf cannot rescue presumably due to the lack of a positive activator and/or substrate(s) that have been sequestered by the mutant protein. However, membrane targeted D-raf rescues, suggesting that it is localized properly and has access to its substrates. However, it is likely that the N-terminal deleted form of D-raf is not localized at the membrane nor does it have access to its substrate(s). D-raf\textsuperscript{S388A} has an access to substrate(s) and is in the right location in D-raf\textsuperscript{A00BB} embryos, but not D-raf\textsuperscript{C2Z2} embryos.

\textit{Ras/Raf/MEK Multiprotein Complex}

Ras and Raf interact when ras is bound to GTP (Moodie et al., 1993; Vojtek et al., 1993). It has been shown that Ras interacts with the N-terminus of Raf (Warne et al., 1993; Zhang et al. 1993; Pumiglia et al., 1995). Van Aelst et al. (1993) showed that the catalytic activity of Raf is not required for interaction with Ras. It has been reported that wild-type Raf interacts with MEK and activates it (Dent et al., 1992; Kyriakis et al., 1992; Macdonald et al., 1993; Jelinek et al., 1994). MEK interacts with the C-terminus of Raf, but does not interact with a Raf ATP binding mutant. Also they found that Ras/Raf/MEK interacted and formed a ternary complex (Huang et al., 1993; Moodie et al., 1993; Van Aelst et al., 1993). Based upon these studies we predict that D-raf\textsuperscript{C2Z2} does not bind ATP nor D-MEK, but will bind activated Ras. On the other hand, D-raf\textsuperscript{A00BB} binds activated Ras and potentially will also bind ATP weakly in the correct orientation. These D-raf molecules would then be able to interact with D-MEK. It has been suggested the active form of Raf-1 associates with MEK and also associates with activated Ras to form a signaling complex (Moodie et al., 1994). We postulate that D-raf acts as a bridge and is necessary to link D-MEK to the membrane.

Constitutively active forms of D-raf do not require activation by the Tor RTK. Therefore, localization and access to substrates are key factors that may limit the
function of constitutively active forms of D-raf. It is likely that D-rafS388A is localized and has a high affinity and access to its substrate(s) in the case of the D-raf400B8 background. In the case of the D-rafC222 background, D-rafS388A is localized, but does not access to its substrate(s) to transfer the signal. This suggests that D-rafC222 proteins occupy a site for D-raf's interaction with its substrate(s), while this site of action becomes available to D-rafS388A in D-raf400B8 background. In the case of N-terminal deleted form of D-raf, it is likely that it may not be able to find D-MEK due to the lack of an N-terminal end that is responsible for targeting D-raf to the membrane.

**Multiprotein Complex Formation for Signal Transduction**

In *Saccharomyces cerevisiae*, mating requires activation of a protein kinase cascade that includes MEJCK, MEK, and MAPK (Errede and Levin, 1993). These three kinases (STEl1, STE7 and FUS3 or KSS1) are associated together in a single complex that is tethered by the Ste5 gene product (Choi et al., 1994). Therefore, STE5 serves as a protein kinase linker. This association is independent of catalytic activity, suggesting that the complex may form prior to signal transduction. Therefore, it is possible that the ternary complex of Ras/Raf/MEK/MAPK can be formed to transduce the signal for the MAPK cascade in *Drosophila*, *C. elegans*, *Xenopus*, and mammals and similar types of regulation for this complex may exist.

**ACKNOWLEDGMENTS**

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REFERENCES


Table 1. Rescue of *D-raf*\(^{11-29}\) mutant embryos by wild-type *D-raf* mRNA: Time dependence

<table>
<thead>
<tr>
<th>Stages</th>
<th>Number of injected embryos</th>
<th>Number of embryos with cuticles*</th>
<th>Rescue of Filzkörper</th>
<th>Percent rescue (%)</th>
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<tbody>
<tr>
<td>Cleavage</td>
<td>26</td>
<td>12</td>
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<tr>
<td>Pole cell formation</td>
<td>16</td>
<td>8</td>
<td>6</td>
<td>75 %</td>
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<tr>
<td>Late syncytial blastoderm</td>
<td>21</td>
<td>13</td>
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<td>23 %</td>
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<td>Cellular blastoderm</td>
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* The male embryos from *D-raf* mutant germlines do not develop cuticles (Materials and Methods).
Table 2. Microinjection of different D-raf mRNAs into D-raf mutant embryos.

<table>
<thead>
<tr>
<th>Maternal genotypes</th>
<th>D-raf mRNA</th>
<th>Number of injected embryos</th>
<th>Number of embryos with cuticles*</th>
<th>Rescue of Filzkörper</th>
<th>Percent rescue</th>
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<tr>
<td>( D-raf^{11-29} )</td>
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<td>86 %</td>
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<tr>
<td></td>
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<tr>
<td>( D-raf^{400B8} )</td>
<td>D-raf(^{WT})</td>
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<tr>
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<td>33</td>
<td>18</td>
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* The male embryos from D-raf mutant germlines do not develop cuticles (Materials and Methods).
Table 3. Microinjection of different D-raf mRNAs into D-raf mutant embryos.

<table>
<thead>
<tr>
<th>Maternal genotypes</th>
<th>D-raf mRNA</th>
<th>Number of injected embryos</th>
<th>Number of embryos with cuticles*</th>
<th>Rescue of Filzkörper</th>
<th>Percent rescue</th>
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<tr>
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<td>D-raf^{S388A}</td>
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<tr>
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<td>D-raf^{raf2}</td>
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<td>42</td>
<td>89 %</td>
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<td></td>
<td>D-raf^{A445}</td>
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<td>100</td>
<td>80</td>
<td>80 %</td>
</tr>
<tr>
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<td>D-raf^{WT}</td>
<td>119</td>
<td>55</td>
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<td>149</td>
<td>88</td>
<td>76</td>
<td>86 %</td>
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<tr>
<td></td>
<td>D-raf^{A445}</td>
<td>152</td>
<td>85</td>
<td>54</td>
<td>64 %</td>
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</table>

* The male embryos from D-raf mutant germlines do not develop cuticles (Materials and Methods).
Figure 1. Expression of D-raf proteins during development. After collected embryos were processed, D-raf immunoprecipitation from embryonic extracts was performed. Then they were resolved by SDS-PAGE (7.5 % polyacrylamide gel) and examined by immunoblotting using a D-raf specific antibody. (A) Maternal expression; Accumulations of 90 kDa D-raf protein in wild-type embryos and 75 kDa D-raf protein in embryos generated from homozygous D-raf^{PB26} germline females. (B) Zygotic expression; Accumulation of 75 kDa D-raf protein in embryos produced by females carrying heterozygous D-raf^{WT}/D-raf^{PB26} on their X-chromosome is shown.
Figure 2. Western blot analysis. Accumulation of D-raf proteins in embryos produced by different D-raf germline clones. (A) wild-type (B) D-raf11-29 (C) D-raf400B8, and (D) D-rafC222.
Figure 3. Induction of terminal structures in $D$-$raf^{400B8}$ embryos after injection of $D$-$raf$ mRNA. Cuticular preparations of embryos are shown after the completion of embryonic development. (A) A wild-type embryo from a wild-type mother with Filzkörper (arrow). (B) An embryo from a homozygous $D$-$raf^{400B8}$ germline female after injection of wild-type $D$-$raf$ mRNA showing no rescue of posterior terminal structures. (C) An embryo from a homozygous $D$-$raf^{400B8}$ germline female after injection of $D$-$raf^{S388A}$ mRNA showing rescue of posterior terminal structures including Filzkörper (arrow).
Figure 4. Western blot analysis. Accumulation of D-raf proteins in embryos produced by different D-raf germline clones. (A) wild-type, (B) D-raf\textsuperscript{raf2}, (C) D-raf\textsuperscript{DF903}, (D) D-raf\textsuperscript{EA75}, and (E) D-raf\textsuperscript{DC817}. 
GENERAL SUMMARY AND CONCLUSIONS

The activation of Torso receptor tyrosine kinase (RTK) and subsequent events in the signal transduction pathway are required for development of terminal structures in *Drosophila* embryos. This pathway relies on the action of maternal products to specify cell fates at the embryonic poles. D-raf, one of maternal products, transmits the Torso (Tor) signal generated at the embryonic poles for the specification of head and tail structures of the embryo.

It has been shown *in vitro* that D-raf has the ability to autophosphorylate when incubated with Mn\(^{2+}\) and \[^{32}\text{P}]\text{ATP}. Phosphoamino acid analysis has revealed that there are phosphoserine and phosphothreonine residues in D-raf after autophosphorylation, indicating that D-raf has intrinsic serine/threonine kinase activity (Sprenger et al., 1993). Thus, as a serine/threonine kinase we predict that the kinase activity of D-raf plays an essential role in transmission of the terminal signal. By microinjecting either wild-type *D-raf* mRNA or kinase defective *D-raf* mRNA into embryos lacking maternal D-raf proteins, we demonstrate that the serine/threonine kinase activity of D-raf is required for the Tor signal transduction pathway and the human Raf-1 kinase can substitute for D-raf kinase in this pathway.

*Regulation of D-raf Serine/Threonine Kinase*

Recently, it has been shown that maternal D-raf becomes phosphorylated two hours after fertilization in wild-type embryos. At this time the Torso RTK has been activated (Sprenger et al., 1993). Furthermore, using phosphopeptide analysis phosphorylation sites in Raf-1 have been mapped (Morrison et al., 1993). In this study, conservation of serine phosphorylation sites was observed between D-raf and mammalian Raf-1 kinases. These findings indicate that the phosphorylation of conserved serine residues is likely an important regulatory mechanism utilized by the Raf family of serine/threonine kinases. Since it has been shown that D-raf has serine/threonine kinase activity, it will be important to find pivotal phosphorylation sites critical for the regulation of its function as a kinase. We find that the serine or its phosphorylation at amino acid 743 is essential for D-raf function in the terminal class pathway, but serine or its phosphorylation at amino
acid 388 is not required for D-raf to transmit the Tor signal. Rather, D-raf\(^{S388A}\) is shown to be constitutively active and rescues the terminal defect in \textit{trunk} mutant embryos in which the Tor receptors are not activated.

The D-raf protein kinase consists of 3 domains: the kinase domain is referred to as CR3; the serine/threonine rich domain, CR2; and the cysteine containing motif, CR1 (Rapp et al., 1988). There is evidence that the amino terminal portion of the protein containing the CR1 and CR2 domains acts to regulate the Raf-1 molecule in mammalian cells. Removal of CR1 and/or CR2 gives rise to an activated Raf-1 molecule (Heidecker et al., 1990; Bruder et al., 1992). Two different truncated forms of D-raf (D-raf\(^{A315}\) and D-raf\(^{A445}\)) are able to rescue the terminal defect in embryos lacking maternal D-raf proteins. This indicates that truncated forms of D-raf in the absence of wild-type D-raf molecules can properly identify its substrate(s) and act to transmit the terminal signal.

Targeting of Raf-1 to the membrane results in a Ras independent, active form of the Raf-1 kinase (Leevers et al., 1994; Stokoe et al., 1994). The membrane targeted form of D-raf not only rescues the terminal defect but suppresses abdominal segmentation. Thus, the membrane associated form of D-raf (D-raf\(^{tor4021}\)), like truncated forms of D-raf, has an access to its substrate(s) and effectively transmits the Tor signal at the posterior pole.

Sprenger and Nüsslein-Volhard (1992) showed that the activation of Tor in the middle of embryos suppresses the normal segmented pattern and promotes ectopic formation of terminal structures. We demonstrate that D-raf\(^{tor4021}\), but not D-raf\(^{S388A}\) or D-raf\(^{A445}\), induces the suppression of abdominal segmentation and development of ectopic Filzkörpere in the middle. Thus, in the middle of the embryo where Tor is not activated, only membrane targeted D-raf promotes terminal development. It is possible that an additional requirement exists for D-raf signaling in the central domain and as a consequence of its membrane localization and amino-terminal truncated sequences, D-raf\(^{tor4021}\) is competent to promote Tor signal transduction within this region. In mammalian cells RTK activation leads to the formation of Ras/Raf-1 complexes at the membrane and this facilitates efficient activation of Raf-1 (Leevers et al., 1994; Stokoe et al., 1994). It has also been demonstrated that Ras, Raf-1, and MEK can form a ternary complex (Jelinek et al., 1994). If D-raf membrane association is required for its biological activity, then it is likely that within the central domain of a \textit{Drosophila} embryo activation of Tor
and/or D-ras1 results in the recruitment of D-raf to the membrane by its interaction with D-ras1. Therefore, we postulate that two requirements for D-raf function are necessary; (1) membrane association of D-raf is essential for its biological function in Tor signal transduction, (2) at the embryonic poles there is a D-raf localizing activity by a "docking protein(s)", that act independent of Tor activation, and (3) this docking protein(s) is absent from the central domain of the embryo.

Interaction between D-raf and Its Activator(s) and Substrate(s)

It has been postulated that the kinase defective Raf-1 competes with endogenous wild-type Raf-1 for a limiting amount of unidentified positive activator (MacNicol et al., 1993). Indeed, endogenous pools of nonfunctional maternal D-raf protein (D-raf\(^{400B8}\) and D-raf\(^{C2Z2}\)) inhibits the rescue of terminal defects by D-raf\(^{WT}\) mRNA, indicating that nonfunctional maternal D-raf protein exhibits a dominant negative effect. This suggests that wild-type D-raf requires an activator that has been sequestered by the maternal mutant D-raf protein.

Accumulation of D-raf protein

We characterized the accumulation of D-raf protein in embryos at 0-2 and 2-4 hours after egg laying, from D-raf mutant germlines. A 90 kDa D-raf protein is detected in wild-type embryos, and those derived from D-raf\(^{400B8}\), D-raf\(^{C2Z2}\), D-raf\(^{DF903}\), and D-raf\(^{437}\) but is absent from D-raf\(^{11-29}\), D-raf\(^{EA75}\), D-raf\(^{107}\), and D-raf\(^{37}\) germlines. Truncated forms of D-raf accumulate in embryos from D-raf\(^{PB86}\) and D-raf\(^{DC817}\) germlines. Thus, there is a correlation between the presence of mutant maternal D-raf protein and the lower lever of rescue by D-raf\(^{WT}\) mRNA injection.

Temporal Regulation of D-raf

The activation of D-raf is required for the expression of the zygotic genes tailless and huckebein (Pignoni et al., 1992). The earliest expression of tailless can be detected at nuclear cycle 11 shortly after pole cell formation (Pignoni et al., 1990, 1992). This indicates that the terminal system is active prior to or at this stage. By
microinjecting wild-type D-raf mRNA into embryos from D-raf^11-29 germline clones at different developmental stages, we find that the temporal control of D-raf activation takes place between nuclear cycle 4 and 12. We also detect that the zygotic expression of the D-raf gene occurs at nuclear cycle 13, 14, and blastoderm stages, indicating that the paternal D-raf protein is not involved in Tor signaling.

Conclusion

These results provide some insights toward understanding the mechanism and regulation of signal transduction processes in Drosophila. It has been shown that several biochemical pathways that function to elicit a developmental and/or mitotic response in mammalian and insect cells require structurally related proteins. Thus, conclusions that are drawn from these studies will not only elucidate the role of D-raf in Drosophila development, but may also provide clues toward understanding the roles played by wild-type and oncogenic Raf-1 in mammalian cells.
LITERATURE CITED


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