The Activity of D-raf in Torso Signal Transduction Is Altered by Serine Substitution, N-Terminal Deletion, and Membrane Targeting

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The Raf family of serine/threonine kinases are essential components in many receptor tyrosine kinase-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 protein, 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 putative serine phosphorylation sites (S388 and S743) in SF9 cells and demonstrate that S743 or its phosphorylation is essential for D-raf function in embryos. Alanine substitution at S388, N-terminal truncation, or targeted membrane association permits transmission of the Torso signal by D-raf, but these D-raf molecules differ in their rescuing potential and relative biological activity. Membrane-targeted D-raf showed the highest level of activity, followed by alanine-substituted D-raf and N-terminal-truncated D-raf. Since the activity profiles for these altered forms of D-raf are distinct, these findings indicate that each structural modification differentially affects the regulation and/or propagation of the Tor signal by these mutant D-raf proteins.

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 (for review see 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 (for review see Lu et al., 1993b). 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., 1989; Brömer and Jäckle, 1991), which 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 (Deegelmann et al., 1986), fs(1) pole hole (Perrimon et al., 1986), torso-like (Stevens et al., 1990; Savant-Bhoné sale and Montell, 1993; Martin et al., 1994), and trunk (Casanova et al., 1995) 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 (see Perrimon, 1993) that include D-ras1 (Lu et al., 1993a) 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 and Zipursky, 1992; Biggs et al., 1994; Brunner et al., 1994). It is through the activity of this phosphorylation cascade that the fate of nuclei at the poles is determined.

How the activity of D-Raf is regulated in this or other RTK-mediated signal transduction pathways, such as the sevenless or EGF-receptor pathways in Drosophila (Dickson et al., 1992; Brand and Perrimon, 1994), is not well understood at this time. Since at the amino acid level there is 45% homology between D-Raf and the human Raf-1 proto-oncogene (Mark et al., 1987; Nishida et al., 1988), regulation of D-Raf's activation and activity is likely similar to that of Raf-1. In mammalian cells, the Raf-1 kinase 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; Pumiglia et al., 1995), 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, 1989). In addition, Raf-1 can associate 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 (Fanti et al., 1994; Freed et al., 1994; Fu et al., 1994; Irie et al., 1994). Furthermore, it has also been demonstrated that Ras, Raf-1, and MEK can form a ternary complex (Huang et al., 1993; Hoang et al., 1994; Moodie et al., 1993; Van Aelst et al., 1993; Jelinek et al., 1994) and is likely to exist as a member of a multiprotein complex(es) (Morrison, 1994). Based upon the complexity of regulation for Raf-1, it is probable that in Drosophila the mechanisms(s) utilized to regulate D-Raf's activity in Tor, as well as in other RTK-mediated signal transduction pathways, is also complex.

D-raf shares with other Raf family members three regions of high amino acid conservation important for its regulation and activity (CR1, CR2, and CR3). CR1, located within the N-terminal domain of the protein, mediates Raf-1 interaction with Ras and its membrane localization. Disruption of CR1 by mutation at Arg29 prevents Raf-1 association with Ras and Ras-mediated activation of Raf-1 (Fabian et al., 1994), while mutation within the cysteine finger motif (C25XCXGXGXC316) at Cys298 attenuates interaction between Raf-1 and Ras (Kolch et al., 1993; Bruder et al., 1992). For D-raf, Arg29 corresponds to Arg211, and mutation of this residue leads to a partial loss of D-raf function (Melnick et al., 1993). CR2 is a region rich in serine and threonine residues and contains Ser259, a major phosphorylation site of Raf-1 (Morrison et al., 1993). Mutation of Ser259 to Ala results in an increase in Raf-1 kinase activity in vitro and prevents its association with 14-3-3 (Michaud et al., 1995). Thus, CR1 and CR2 appear to regulate Raf-1 function and activity; their deletion activates the transforming activity of Raf-1 (Stanton et al., 1989; Heidecker et al., 1990). CR3 is the substrate binding and kinase domain of the protein. Alteration of Ser227, an additional site of phosphorylation conserved in all Raf family members, is essential for Raf-1 activity (Morrison et al., 1993; Fabian et al., 1993).

We have utilized the Tor pathway to learn about the regulation of D-raf and its signal transduction by microinjection of D-raf mRNAs into Drosophila embryos. As a single large cell at early stages of embryogenesis, the Drosophila embryo serves as an in vivo system in which to assay the activity and biological function of various D-raf proteins. In the absence of D-raf-mediated Tor signal transduction, the embryonic head and tail fail to develop. Rescue of this defect by microinjection of wild-type and mutant D-raf mRNAs is used to assess whether propagation of the Tor signal has been achieved by these forms of D-raf. Here, we examine their effects 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 in Sf9 cells, N-terminal truncations, a membrane-associated D-raf fusion protein, and 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 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 these embryos. In addition, we identified two putative sites of D-raf phosphorylation in Sf9 cells and show that one, serine S743, is essential for D-raf function in the Tor pathway. Alternatively, alanine substitution at residue S388 (D-raf388A) did not inhibit D-raf's ability to act in Tor signal transduction. Also, we show that N-terminal-truncated D-raf313, D-raf370, D-raf445, and a membrane-targeted D-raf4021 can participate in Tor signaling. However, the biological activities of these mutant D-raf proteins, including D-raf388A, are between 4- and 380-fold greater than wild type, indicating that these structural modifications may distinctly alter the regulation and/or propagation of the Tor signal by D-raf.

MATERIALS AND METHODS

Drosophila Strains

In this study, wild-type Oregon R (Lindsley and Zimm, 1992) and the D-raf12-29 allele (Ambrosio et al., 1989b) were used. To generate germ line clones of D-raf12-29, the “FLP-DFS” technique was utilized (Chou and Perrimon, 1992). Flies were raised on Drosophila medium at 25°C under standard conditions (Roberts, 1986; Wieschaus and Nüsslein-Volhard, 1986; Ashburner, 1989).

Microinjections

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 nuclear cycle (NC) 6–9 were used for injection, and staging was according to Foe and Alberts (1983) and Campos-Ortega and Hartenstein (1985). After 48
hr at 19°C, embryos were devitellinized with a sharp tungsten needle and Halocarbon oil (series 95, Halocarbon Products Corp.) 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.

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 our analysis, we address the question of posterior rescue for these female embryos. Thus, since the D-raf protein product acts in several different developmental pathways, only its maternal role in the Tor pathway has been addressed here.

Plasmid Constructions

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

Site-directed mutagenesis. The plasmid pGEM7GLO was generated by inserting a 2.7-kb HindIII–EcoRI fragment from pSP64GLO, which contained the entire coding sequence of the D-raf gene (residues 1–782) inserted 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′-CCCGTGCGCTTAATGACACTCAACGTTG-3′), S389A (5′-CAAGAGCGATCGATCC-3′), and S743A (5′-CATCGCAGTGCCGCAAAATTG-3′), and S743A (5′-CATCGCAGTGCCGCAAAATTG-3′). Mutations were generated by the method of Kunkel et al. (1987), as described by the vendor (for Bio-Rad Mutagenesis Kit, Richmond, CA), and each mutation was confirmed by sequencing using a custom primer and the Sequenase Western blot analysis. After injection, the embryos were incubated for 1 hr at 37°C and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Expression of D-raf Proteins in Sf9 Cells

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

Phosphopeptide Mapping

For peptide mapping, 32P-labeled proteins isolated from Sf9 cells were separated by 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 1000 V, followed by ascending chromatography in a buffer containing n-butanol:pyridine:acetic acid:water in a ratio of 75:50:15:60.

Western Analysis of D-raf from 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 at a concentration of 1 embryo per 1 µl (see Sprenger et al., 1993). Insoluble material was removed by centrifugation at 4°C for 10 min at 10,000g.

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 hr, washed in TMB (TBS containing 0.2% Tween 20), and probed for 2 hr with primary antibody diluted in TBS. The filters were then washed in TBS, incubated with a horseradish peroxidase-coupled secondary antibody for 1 hr, and washed again in TBS. They were developed by using an enhanced chemiluminescence system (Amersham) and visualized on Kodak XAR-5 film.
FIG. 1. Linear representations of the primary amino acid structure for wild-type and modified D-raf proteins. CR1 contains the putative D-ras1 binding domain and a cysteine zinc finger-like motif, 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 and extracellular and transmembrane domains of the Tor protein are depicted. EC, extracellular domain; TM, transmembrane domain.

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-rafWT) or a kinase-defective mutant D-raf (D-rafK497M) into embryos lacking maternal D-raf protein. These embryos were derived from mothers homozygous mutant for the D-raf11-29 allele in their germlines (Chou and Perrimon, 1992). Figure 1 shows a schematic representation of the wild-type D-raf and the mutant D-raf proteins used in this analysis. mRNA was injected at a concentration of approximately 1 μg/μl since this insured levels of D-raf protein as high as those found in wild-type embryos. After injection of D-rafWT or D-rafK497M mRNA, embryos were allowed to develop for 24–48 hr, and analyzed for the presence of terminal structures including Filzkörper.

Figure 3 shows cuticular preparations of a wild-type embryo (Fig. 3A) and mutant embryos that lack maternal D-raf protein from females with homozygous D-raf11-29 germlines (Figs. 3B–3F). In the absence of maternal D-raf, head and tail structures are not formed (Figs. 3B and 3E). After posterior injection of D-rafWT mRNA, rescue of pattern including Filzkörper, tuft, and anal pads was observed (Fig. 3C). Rescue of head structures was achieved when D-rafWT mRNA was injected into the head region (Figs. 3D and 3F). In contrast, injection of D-rafK497M 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

FIG. 2. 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. (A) Accumulation of 90-kDa D-raf protein in wild-type embryos. (B) No accumulation of D-raf protein is observed after injection of distilled water into embryos derived from females with homozygous D-raf11-29 germlines. (C–F) Accumulation of D-raf protein in embryos from D-raf11-29 female germlines after injection of increasing 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.
FIG. 3. 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-raf11-29 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-raf11-29 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. db, dorsal bridge; vpp, ventral posterior process.

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 T or signaling pathway.

### TABLE 1

<table>
<thead>
<tr>
<th>Region (egg length)</th>
<th>D-raf mRNA</th>
<th>No. of injected embryos</th>
<th>No. of embryos with cuticles</th>
<th>Rescue of Filzkörper or medial tooth</th>
<th>Percentage rescue (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Posterior (90–100%)</td>
<td>wild type</td>
<td>120</td>
<td>57</td>
<td>50</td>
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<tr>
<td>Anterior (0–10%)</td>
<td>wild type</td>
<td>137</td>
<td>75</td>
<td>0</td>
<td>0%</td>
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<tr>
<td></td>
<td>K497M</td>
<td>199</td>
<td>75</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td>K497M</td>
<td>178</td>
<td>64</td>
<td>65</td>
<td>87%</td>
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</tbody>
</table>

*Male embryos from D-raf mutant germlines do not develop cuticles. Percentage rescue refers only to those female embryos with cuticles (see Materials and Methods).
TABLE 2
Rescue of Embryos That Lack Maternal D-raf Protein by Wild-Type D-raf mRNA: Time Dependence

<table>
<thead>
<tr>
<th>Nuclear cycle (NC) stages</th>
<th>No. of developed embryos</th>
<th>No. of embryos with cuticles&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Rescue of Filzkörper</th>
<th>Percentage rescue (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC 6-8</td>
<td>26</td>
<td>12</td>
<td>10</td>
<td>83%</td>
</tr>
<tr>
<td>NC 9-10</td>
<td>16</td>
<td>8</td>
<td>6</td>
<td>75%</td>
</tr>
<tr>
<td>NC 12-13</td>
<td>21</td>
<td>13</td>
<td>3</td>
<td>23%</td>
</tr>
<tr>
<td>NC14</td>
<td>20</td>
<td>10</td>
<td>0</td>
<td>0%</td>
</tr>
</tbody>
</table>

<sup>a</sup> The male embryos from D-raf mutant germlines do not develop cuticles. Percentage rescue refers only to those female embryos with cuticles (see Materials and Methods).

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 gene expression begins about 60 min 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-raf<sup>WT</sup> mRNA at 1 μg/μl was injected into embryos that lack maternal D-raf protein. When embryos were injected at NC 6-8 or NC 9-10, 83 and 75% of the embryos developed Filzkörper, respectively (Table 2). However, at NC 12-13 the percentage rescue was dramatically reduced (23%) and at NC 14 after the onset of cellularization none of the injected embryos developed Filzkörper. Sprenger and Nüsslein-Volhard (1992) observed a similar age-dependent rescue of tailless mutant embryos after the injection of tor mRNA. This experiment indicates that the optimal period of activation and signal transduction by D-raf in the Tor pathway is between NC 6 and 12. For the experiments described below, mRNAs were injected into embryos at stages NC 6-9 of embryonic development.

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

Since there is 45% amino acid homology between D-raf and Raf-1 (Mark et al., 1987; Nishida et al., 1988), we tested the ability of human Raf-1 to act in the Drosophila Tor transduction pathway. In this experiment, 1 μg/μl 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 pathway. We also tested whether a highly transforming N-terminal deletion of human Raf-1, Raf-1<sup>1-305</sup> (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 after injection of 1 μg/μl Raf-1<sup>1-305</sup> mRNA. These results indicate that there is conservation of structure among 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 2 hr 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 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 [32P]orthophosphate in Sf9 cells. Since there is 45% amino acid homology between D-raf and Raf-1, 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 (Fig. 4). For D-raf<sup>WT</sup> five labeled peptides were observed (Figs. 4A and 4D). For the altered D-raf proteins D-raf<sup>S388A</sup> (Fig. 4B) and D-raf<sup>S743A</sup> (Fig. 4C), phosphopeptides A and B, respectively, were absent. This analysis indicates that serine residues 388 and 743 serve as putative D-raf phosphorylation sites in Sf9 cells and suggests that these sites may also be phosphorylation sites in the Drosophila embryo. In addition, since the phosphopeptide map of kinase-defective D-raf<sup>K497M</sup> is identical to that of D-raf<sup>WT</sup>, it is unlikely that the phosphorylated peptides result from D-raf autophosphorylation (data not shown).
FIG. 4. Two dimensional phosphopeptide maps of wild-type and D-raf proteins with serine to alanine substitutions. In vivo $^{32}$P-labeled D-raf proteins isolated from Sf9 cells were digested with trypsin and resolved in two dimensions on TLC plates by electrophoresis (vertical axis) and by chromatography (horizontal axis). (A) For D-raf$^{WT}$ five labeled phosphopeptides, depicted as phosphopeptides A–E (D), are observed. (B) For D-raf$^{S388A}$ labeled phosphopeptide B is absent. (C) For D-raf$^{S743A}$ phosphopeptide A is absent while those for B (arrow) and C–E are present. (D) Schematic representation of the five wild-type D-raf phosphopeptides.

### 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, 1 μg/μl 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 (see Fig. 1). After 48 hr embryos were scored for the presence of Filzkörper (Table 3). After injection with D-raf$^{S388A}$ mRNA, rescue of posterior pattern was observed (Fig. 5A) at a level similar to that of D-raf$^{WT}$ mRNA (78 compared to 88%). However, D-raf$^{S743A}$ (Fig. 5B) 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 negative regulation (Heidecker et al., 1990). We tested whether D-raf proteins

### Table 3

<table>
<thead>
<tr>
<th>D-raf mRNA</th>
<th>No. of injected embryos</th>
<th>No. of embryos with cuticles</th>
<th>Rescue of Filzkörper</th>
<th>Percentage rescue (%)</th>
</tr>
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<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>
<tr>
<td>D-raf$^{315}$</td>
<td>153</td>
<td>86</td>
<td>49</td>
<td>57%</td>
</tr>
<tr>
<td>D-raf$^{370}$</td>
<td>120</td>
<td>68</td>
<td>37</td>
<td>54%</td>
</tr>
<tr>
<td>D-raf$^{344S}$</td>
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<td>90</td>
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<tr>
<td>D-raf$^{6021}$</td>
<td>85</td>
<td>51</td>
<td>47</td>
<td>92%</td>
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</table>

$^a$ Male embryos from D-raf mutant germlines do not develop cuticles (Materials and Methods).
having N-terminal truncations would substitute for wild-type D-raf in the Tor pathway (see Fig. 1). For this analysis three deletions of D-raf were tested, D-raf<sup>D315</sup>, D-raf<sup>D370</sup>, and D-raf<sup>D445</sup>. D-raf<sup>D315</sup> lacks CR1, which contains the putative Ras-1 binding domain and the cysteine finger region. D-raf<sup>D370</sup> lacks CR1 and part of CR2, but retains the rest of CR2 and the serine/threonine kinase domain (CR3). D-raf<sup>D445</sup> encodes only CR3. As shown in Table 3, when 1 μg/μl of each of these deleted forms of D-raf mRNAs was injected into the posterior of embryos lacking maternal D-raf protein, greater than 50% rescue of Filzkörper was achieved. Figures 5C and 5D show two embryos that lack maternal D-raf protein after injection of D-raf<sup>D370</sup> and D-raf<sup>D445</sup> mRNAs, respectively. Both embryos show rescue of posterior pattern. Rescue of head structures was also observed after anterior injection of D-raf<sup>D315</sup>, D-raf<sup>D370</sup>, and D-raf<sup>D445</sup> mRNAs (data not shown). These results indicate that N-terminal-truncated D-raf molecules can recognize and interact with their substrate(s) to transmit the Tor signal in embryos.

It has also been 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 tested a deletion mutation, D-raf<sup>tor4021</sup>, which encodes the CR2 and CR3 sequences equivalent to D-raf<sup>D370</sup> fused to the signal sequence and extracellular and transmembrane domains of the Tor protein (see Fig. 1). In D-raf<sup>tor4021</sup>, 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). This fusion protein has 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).

When 1 μg/μl of D-raf<sup>tor4021</sup> mRNA was injected into the posterior of embryos that lack maternal D-raf protein, rescue of posterior Filzkörper was observed. However, in these experiments many of the embryos produced weak cuticles that ruptured during the process of mounting. Other embryos had cuticles that appeared faint or crinkled in which terminal as well as abdominal structures were difficult to score. Therefore, we tested the effect of microinjection using 0.25 μg/μl of D-raf<sup>tor4021</sup> mRNA. As shown in Table 3, rescue of Filzkörper was observed for 92% of the injected embryos.

**FIG. 5.** Development of D-raf embryos that lack maternal D-raf protein after posterior injection of modified D-raf mRNAs. Cuticular preparations of embryos are shown after the completion of embryonic development. (A) Rescue of posterior Filzkörper (arrow) after injection of D-raf<sup>D388A</sup> mRNA. (B) Terminal rescue is not observed after injection of D-raf<sup>D324A</sup> mRNA. (C) Rescue of posterior Filzkörper (arrow) after injection of D-raf<sup>D335</sup> mRNA. (D) Rescue of posterior Filzkörper (arrow) after injection of D-raf<sup>D445</sup> mRNA. (E) Rescue of posterior Filzkörper (arrow) after injection of D-raf<sup>tor4021</sup> mRNA. (F) For some embryos after injection of D-raf<sup>tor4021</sup> mRNA the region containing the anal plates was enlarged (arrow) and this expansion was accompanied by deletion of part or all of abdominal segment eight.
D-raf in Torso Signal Transduction

When injected intracellularly into embryos that lack maternal D-raf protein, D-raf mRNA led to only 10% rescue of Filzkörper observed. Thus, it appears that there is a threshold level of wild-type activity required to achieve a high degree of terminal rescue. To determine the relative biological activity of wild-type and mutant D-raf mRNAs, we calculated the approximate concentration of mRNA needed for injection to achieve 80% terminal rescue. For D-raf WT approximately 0.76 μg/μl mRNA would be required. Similarly, to achieve 80% rescue the approximate concentration of mRNA required for injection of D-raf S388A is 0.097 μg/μl mRNA, while for D-raf D445 0.030 μg/μl mRNA is required, and for D-raf tor4021 0.002 μg/μl mRNA is required (Fig. 6). Using these calculations, the biological activity of D-raf D445 mRNA is approximately 7.5-fold greater than wild-type, while D-raf S388A and D-raf tor4021 embryos, and very few embryos produced weak cuticles. An example of a rescued embryo is depicted in Fig. 5E. Interestingly, a second effect of D-raf tor4021 was observed, and this is shown in Fig. 5F (arrows). For some embryos the region containing the anal plates was enlarged and this expansion was accompanied by deletion of part of all of abdominal segment eight, which is also specified by the Tor pathway. Thus, unlike wild-type, D-raf S388A, or D-raf D445 proteins, expression of D-raf tor4021 in some embryos directed the formation of an altered posterior pattern. Rescue of head structures was also observed after anterior injection of D-raf tor4021 mRNA (data not shown).

Relative Biological Activity of Wild-Type and Mutant D-raf mRNAs

Since serine substitution at S388A, amino-terminal truncation, and membrane targeting may affect regulation of and signaling by D-raf, we determined the relative biological activity and rescuing potential of wild-type, D-raf S388A, D-raf D445, and D-raf tor4021 mRNAs (Fig. 6). In these experiments a similar volume of mRNA was injected into each embryo and at each mRNA concentration more than 50 embryos with developed cuticles were scored. For wild-type mRNA, posterior rescue was the highest after injection of 1 μg/μl mRNA (99%). At lower mRNA concentrations the percentage of embryos that showed posterior rescue declined. After injection of approximately 0.71 and 0.50 μg/μl wild-type D-raf mRNA, 76 and 70% rescue was achieved, respectively, while at approximately 0.36 μg/μl only 10% rescue of Filzkörper was observed. Thus, it appears that there is a threshold level of wild-type activity required to achieve a high degree of terminal rescue. To determine the relative biological activity of wild-type and mutant D-raf mRNAs, we calculated the approximate concentration of mRNA needed for injection to achieve 80% terminal rescue. For D-raf WT approximately 0.76 μg/μl mRNA would be required. Similarly, to achieve 80% rescue the approximate concentration of mRNA required for injection of D-raf S388A is 0.097 μg/μl mRNA, while for D-raf D445 0.030 μg/μl mRNA is required, and for D-raf tor4021 0.002 μg/μl mRNA is required (Fig. 6). Using these calculations, the biological activity of D-raf D445 mRNA is approximately 7.5-fold greater than wild-type, while D-raf S388A and D-raf tor4021

FIG. 6. Comparison of the relative biological activity of wild-type and mutant D-raf mRNAs. Percentage posterior rescue for embryos that lack maternal D-raf protein after injection of D-raf WT, D-raf D445, D-raf S388A, or D-raf tor4021 mRNA at various mRNA concentrations. A similar volume of mRNA was injected into each embryo and at each mRNA concentration more than 50 embryos with developed cuticles were scored.

FIG. 7. Accumulation of D-raf protein produced in vivo after microinjection of D-raf mRNAs into embryos that lack maternal D-raf protein. For each sample, 100 embryos were injected, allowed to develop for 60 min, and then processed for Western analysis. (A) Western blot of D-raf WT, D-raf S388A, D-raf D445, and D-raf tor4021 proteins that accumulate after injection of 1 μg/μl of the corresponding D-raf mRNA. By comparison, the accumulation of D-raf WT is approximately one-half, that of D-raf D445 is approximately twice, and that of D-raf tor4021 is approximately equivalent to the level observed for D-raf WT. (B) Accumulation of D-raf tor4021 protein after injection of 0.125, 0.25, 0.50, and 1.0 μg/μl D-raf tor4021 mRNA. Densitometric analysis was performed using NIH Image 1.56 (see Materials and Methods).
mRNAs are 25- and 380-fold more active, respectively, than wild-type D-raf mRNA.

Since less than wild-type levels of mRNA were required to achieve a high percentage of embryonic rescue, the concentration of each mutant protein produced in vivo was determined. Figure 7A shows the results of this experiment, in which embryonic lysates were compared after injection of 1 μg/μl D-raf WT, D-raf S388A, D-raf S388A, or D-raf S388A mRNA. For each sample, 100 embryos were injected, allowed to develop for 60 min, and then processed for Western analysis. The relative level of each mutant protein synthesized was determined by comparing its level of accumulation to that found after injection of 1 μg/μl wild-type D-raf mRNA. For D-raf S388A, a 90-kDa D-raf protein accumulates at a concentration of approximately one-half that observed for the wild-type D-raf protein. After injection of D-raf S388A mRNA, a truncated D-raf protein less than 45 kDa was produced at a level 2-fold greater than that found for wild-type D-raf. Finally, as predicted, a greater than 116-kDa protein accumulated after injection of D-raf S388A mRNA at a level approximately equal to that found for D-raf WT protein. In Fig. 7B, we also show that after injection of 0.125–1.0 μg/μl of D-raf S388A mRNA there was a gradual increase in the amount of D-raf S388A protein synthesized in vivo. Similar results were achieved for D-raf S388A and D-raf S388A mRNAs (data not shown). Thus, in these experiments an equivalent amount of D-raf S388A, one-half the quantity of D-raf S388A, and 2-fold more D-raf S388A protein accumulated compared to wild-type. If protein concentration is then taken into consideration when calculating biological activity, D-raf S388A mRNA is 380-fold more active, D-raf S388A mRNA is 50-fold more active, and D-raf S388A mRNA is four times as active as wild-type D-raf mRNA.

DISCUSSION

The Tor pathway is activated when the Drosophila embryo is a syncytium. 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 mutant D-raf proteins and 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 kinase 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 for transmission of cellular signals (see Perrimon, 1993).

In general, signal transduction begins following the binding of ligand molecules to receptor tyrosine kinases that dimerize, leading to their transphosphorylation on tyrosine residues (see van der Greer et al., 1994). These phosphorylated molecules then serve as attachment sites for the adapter protein, Drk, which recruits the guanine nucleotide-releasing factor Son of Sevenless (SOS) to form the membrane-associated activating complex for Ras. In turn, Ras promotes Raf membrane association, and this complex phosphorylates and activates MEK, a protein kinase with dual specificity (see Marshall, 1995). MEK then activates MAPK, which is then translocated to the nucleus where it phosphorylates transcription factors that regulate gene expression. In the case of the Tor pathway, an unknown transcription factor(s) is responsible for expression of the terminal zygotic genes tailless and huckebein (see Lu et al., 1995). Additional complexity in Tor signaling has been reported by Hou et al. (1995), because activation of D-raf by Tor can occur in a D-ras1-independent manner.

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 Mn²⁺ and [γ-³²P]ATP (Sprenger et al., 1993). In addition, Melnick et al. (1993) showed that two mutations in D-raf that gave a null phenotype in the Tor pathway were associated with single amino acid substitutions within conserved regions of the D-raf 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, 1990). Here we show directly that a point mutation known to inactivate 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 in vivo. Thus, D-raf’s kinase activity is essential for D-raf function in Tor signal transduction.

Conservation of Structure and Function of Signal Transduction Molecules

In order to test the evolutionary conservation of the Tor signal transduction pathway, we tested whether human Raf-1 could substitute for D-raf in Drosophila embryos. Full-length human Raf-1 replaced D-raf in the Tor pathway at low efficiency, but the highly transforming N-terminal-deleted form of Raf-1 was twice as active. 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. Therefore, 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. Indeed, it has recently been shown that reversal of Raf-1 activation is achieved by purified and membrane-associated protein phosphatases (Dent et al., 1995). Two putative phosphorylation sites have been mapped for the D-raf kinase isolated from Sf9 cells, serine residues S388 and S743. These two residues and surrounding residues (RSXSXP) are conserved among members of the Raf family of protein kinases and correspond to serines 259 and 621 of the Raf-1 protein (Morrison et al., 1993). Since phosphorylation occurs at these sites for the kinase-defective D-raf<sup>497TM</sup> protein, these events are not due to autophosphorylation and appear to be dependent upon another kinase(s). These results suggest that in Drosophila embryos, 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 serine 388 was permissive and resulted in rescue of the D-raf maternal effect. This result is consistent with that observed for Raf-1 when the homologous amino acid substitutions were tested. In in vitro kinase assays Raf-1<sup>S259A</sup> had no activity, while Raf-1<sup>S259A</sup> showed a threefold increase in kinase activity compared to that of wild-type Raf-1 (Morrison et al., 1993). Unlike wild-type Raf-1, Raf-1<sup>S259A</sup> did not associate with 14-3-3 and showed Ras-independent enzymatic activity as assayed by the induction of meiotic maturation in Xenopus oocytes (Michaud et al., 1995). It is likely that functional conservation at these two phosphorylation sites is indicative of an evolutionarily conserved mechanism that modulates Raf activity. Thus, 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**

In mammalian cells, N-terminal truncation of Raf-1 leads to increased Raf-1 kinase activity and oncogenic transformation (Stanton et al., 1989; Heidecker et al., 1990). Here, we tested whether truncated versions of D-raf can substitute for full-length Raf and transmit the Tor signal in embryos that lack maternal D-raf protein. We tested three different N-terminal deletions, each encoding the D-raf kinase domain, that effectively participated in signal transduction. These results indicate 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. This result is significant because we show that truncated forms of D-raf can act in the absence of and independent of wild-type D-raf. Thus, oncogenic forms of Raf-1 may act in a similar manner in mammalian cells that contain wild-type Raf-1 proteins.

Next, we addressed the effect on Tor signal transduction of a membrane-targeted N-terminal-truncated D-raf. In mammalian cells, targeting of 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). D-raf<sup>tor4021</sup>, a chimeric D-raf protein used in this study, is directed to the membrane through the Tor signal sequence and was shown to induce R7 development in the Sevenless/D-ras1 signal transduction pathway in the absence of Sevenless RTK activity (Dickson et al., 1992). Thus, this chimeric form of D-raf probably has constitutive activity in all cell types in which it is expressed. Here we test whether D-raf<sup>tor4021</sup> can substitute for wild-type D-raf and transmit the Tor signal in the absence of maternal D-raf protein. We find that D-raf<sup>tor4021</sup> promotes formation of terminal structures and at high concentrations can cause the expansion of some posterior elements within the terminal domain. This result indicates that higher than wild-type levels of D-raf activity are produced by D-raf<sup>tor4021</sup> and is consistent with the idea that D-raf<sup>tor4021</sup> acts constitutively in the Tor signaling pathway.

**Relative Biological Activity of Wild-Type and Mutant D-raf mRNAs**

The maternal terminal system defines the spatial expression patterns of the tailless (tll) and huckebein (hkb) genes. Transcriptional activation of tll requires a low level of D-raf activity and occurs in nuclei that lie within the presumptive tail region, at 0–20% egg length. High levels of D-raf activity promote hkb expression and are restricted to nuclei at 0–12% egg length (Melnick et al., 1993). In this study we define rescue of the Tor pathway by scoring development of Filzkörper that require tll, but not hkb expression (Weigel et al., 1989). However, many of the rescued embryos contain pattern elements that are induced by hkb expression (Baek and Ambrosio, unpublished data). Although we assay biological function of D-raf simply at the activity level that triggers tll expression, in many embryos it is probable that expression of hkb is also induced.

When the biological activity of D-raf<sup>MT</sup> was characterized at low concentrations of D-raf mRNA (0.36 μg/μl), 10% of the injected embryos developed Filzkörper. However, at a slightly higher concentration (0.5 μg/μl) 72% rescue of Filzkörper was observed. This indicates that there is a critical threshold of D-raf activity required to promote Filzkörper formation. At twice this concentration (1.0 μg/μl) 99% of the embryos developed Filzkörper. We designated 80% rescue as a good measure of D-raf biological function and then
compared the concentration of wild-type and modified forms of D-raf mRNA required to achieve this level of rescue.

When the relative biological activity of each modified form of D-raf was determined in vivo and normalized for protein concentration, the activity of D-raf<sup>tor4021</sup> mRNA was 380-fold greater, D-raf<sup>S388A</sup> mRNA was 50-fold more active, and D-raf<sup>445</sup> mRNA was four times as active as wild-type D-raf mRNA. These differences in biological activity levels are likely related to the structural and conformational characteristics of each modified form of D-raf. Regulation in Tor signaling involves activation and likely the inactivation of D-raf's kinase activity. Distinct from its function as a kinase, the assembly of D-raf into a multiprotein complex(es) may also prove to be an important mechanism(s) that provides positive and/or negative control, as is the case for Raf-1 and its association with Ras, 14-3-3, hsp 90, and p50 proteins (see Morrison, 1994). Thus, the higher than wild-type activity levels observed for D-raf<sup>tor4021</sup>, D-raf<sup>S388A</sup>, and D-raf<sup>445</sup> are likely a reflection of their release from negative control normally provided by kinase inactivation and/or obligatory protein interactions required for the activation process or for stability of the active enzyme.

At similar mRNA concentrations, D-raf<sup>tor4021</sup> is much more active at promoting development of Filzkörper than either D-raf<sup>S388A</sup> or truncated D-raf<sup>445</sup>. It is likely that this high level of D-raf<sup>tor4021</sup> activity is related to the membrane targeting sequence of the chimeric truncated D-raf protein. One possible explanation for this finding is that by putting D-raf directly onto the membrane, requirements for upstream proteins in the pathway are bypassed. Also, D-raf<sup>tor4021</sup> may have better access to its substrates, thereby transmitting the signal with greater efficiency. Both D-raf<sup>S388A</sup> and D-raf<sup>445</sup> may require interaction with upstream components to transmit the signal and thus be dependent upon the number of sites available through Tor signaling. It is not clear whether the substrate(s) of D-raf is localized to the membrane or is free to diffuse in the cytoplasm. In yeast, a multiprotein kinase complex is tethered by Ste7, which contains the MAP kinase kinase kinase (Ste11), MAP kinase kinase (Ste7), and MAP kinase (Fus3/Kss1) homologues (Choi et al., 1994; Krantz et al., 1994). It is unknown whether a similar multiprotein kinase complex that contains D-raf/D-MEK/MAP kinase must form before the Tor signal can be transduced.

Alternatively, biological activity may simply depend upon the concentration of D-raf molecules at the membrane. For D-raf<sup>tor4021</sup>, all of the molecules synthesized are presumably anchored in the membrane as a result of the Tor signal sequence. However, D-raf<sup>S388A</sup> and D-raf<sup>445</sup> are likely evenly distributed throughout the cytoplasm. Thus, at the membrane the local concentration of D-raf<sup>tor4021</sup> is likely greater than that of the nonmembrane-targeted D-raf<sup>S388A</sup> or D-raf<sup>445</sup> molecules. Thus, the absolute biological activity per molecule of D-raf<sup>tor4021</sup> may be no greater than that of D-raf<sup>S388A</sup> or D-raf<sup>445</sup>. By targeting D-raf<sup>S388A</sup> as well as wild-type D-raf protein to the membrane, this question may be addressed. Further characterization of these modified forms of D-raf in embryos lacking the activity of upstream pathway components will provide additional clues toward understanding the mechanisms utilized in wild-type cells to promote transduction of a signal from the membrane to the nucleus.

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