Drosophila-Raf Acts to Elaborate Dorsoventral Pattern in the Ectoderm of Developing Embryos

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ABSTRACT

In the early Drosophila embryo the activity of the *EGF-receptor* (*Egfr*) is required to instruct cells to adopt a ventral neuroectodermal fate. Using a gain-of-function mutation we showed that *D-raf* acts to transmit this and other late-acting embryonic *Egfr* signals. A novel role for *D-raf* was also identified in lateral cell development using partial loss-of-function *D-raf* mutations. Thus, we provide evidence that zygotic *D-raf* acts to specify cell fates in two distinct pathways that generate dorsoventral pattern within the ectoderm. These functional requirements for *D-raf* activity occur subsequent to its maternal role in organizing the anterioposterior axis. The consequences of eliminating key *D-raf* regulatory domains and specific serine residues in the transmission of *Egfr* and lateral epidermal signals were also addressed here.

As a member of the Ras/mitogen-activated protein kinase (MAPK) signaling cassette, the *D-raf* serine/threonine kinase plays an essential role in numerous developmental pathways in Drosophila. In the early embryo, D-raf proteins transmit a cell determination signal localized at the embryonic poles that depends on the activation of the Torso (Tor) receptor tyrosine kinase (RTK). Both *D-raf* and *tor* mRNAs are maternally synthesized and translated into proteins that specify terminal cellular fates within 3 hr after egg fertilization (reviewed by Lu *et al.* 1993; Duffy and Perrimon 1994). Subsequently, along the ventral midline, a second RTK-generated signaling pathway, under the control of the Drosophila *EGF receptor* (*Egfr*), results in the specification of ventral ectodermal cell fates (reviewed by Schweitzer and Shilo 1997).

It has been anticipated, but not previously shown, that *D-raf* acts as an essential component for transmission of the *Egfr*-generated signal in ventral ectodermal cells of the embryo. Other embryonic *Egfr* pathways in which *D-raf* may function include those required for germband retraction, the development and viability of midline glial cells, and the secretion of ventral cuticle and denticles (Clifford and Schüpbach 1992; Raz and Shilo 1992, 1993; Scholz *et al.* 1997). At later stages of the life cycle, it has been shown that *D-raf* functions downstream of *Egfr* in wing vein differentiation (Diaz-Benjumea and Hafen 1994), specification of photoreceptor cells (Greenwood and Struhl 1999; Halfar *et al.* 2001; Yang and Baker 2001), and dorsoventral patterning of follicle cells in the ovary (Brand and Perrimon 1993).

It is known that the regulation of the *Raf* kinase family is complex, with a variety of proteins acting to control subcellular localization, conformational state, and ultimately the kinase activity of Raf molecules (for review and references within see Kolch 2000). *Raf* family members share homology in three domains, conserved region 1 (CR1) that binds Ras, CR2 that binds 14-3-3, and CR3, the kinase region that also contains a 14-3-3 binding site. In mammals, Ras acts positively to position Raf-1 at the membrane, where it is subsequently activated (Leevers *et al.* 1994; Stokoe *et al.* 1994). In contrast, the phospho-binding protein, 14-3-3, has been implicated in both the positive and negative regulation of Raf-1 functioning as a "scaffold" that stabilizes both the active and inactive forms of the kinase (Michaud *et al.* 1995; Muslin *et al.* 1996).

In Drosophila, *D-Ras* acts to positively regulate the activity of *D-raf* in *Tor*, *Egfr*, and *Sevenless* signaling pathways (reviewed by Daum *et al.* 1994; Duffy and Perrimon 1994). In addition, two isoforms of 14-3-3 (ε and ζ) have been shown to enhance *D-Ras* signaling and are thought to operate through interaction with D-raf proteins (Chang and Rubin 1997; Kockel *et al.* 1997; Li *et al.* 1997). Since 14-3-3 ζ has also been implicated in the negative regulation of *D-raf* (Rommel *et al.* 1997) it is likely that the 14-3-3 proteins can, in a manner equivalent to that found for Raf-1, bind to D-raf S388 and S743 to regulate activity. However, it is clear from genetic studies that other factors, including KSR (Therrien *et al.* 1995, 1996), PP2A (Wassarman *et al.* 1996), and CNK (Therrien *et al.* 1998, 1999) also serve to modulate the activity of *D-raf* (for review see Sternberg and Alberola-Ila 1998).

Signal transduction pathways mediate cellular responses such as growth and differentiation by eliciting a signal across the plasma membrane. The mechanisms that regulate *Raf* family members in different organisms

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in these signaling pathways during development remain unclear. Since the family of *Raf* proteins has been highly conserved during evolution, studies involving *D-raf* will have broad implications and better define how developmental cell fate choices are generated within the animal kingdom. In *Caenorhabditis elegans* the *lin 45 raf* kinase is required for vulva development (Han *et al.* 1993). In *Xenopus laevis* Raf proteins have been shown to play a role in the induction of the embryonic mesoderm (MacNicol *et al.* 1993, 1995; Xu *et al.* 1996). *Raf-1*-deficient mice exhibit growth retardation and die at midgestation with anomalies in the placenta and in the fetal liver (Naumann *et al.* 1997; Mikula *et al.* 2001), while *B-Raf*-deficient mice die *in utero* displaying defects in endothelial cell differentiation and survival (Wojnowski *et al.* 1997).

Here we access the ability of D-raf molecules to act in signaling pathways activated subsequent to the establishment of terminal cell fates by the Tor RTK. We found that mutant forms of *D-raf* expressed using a heat-shock-driven transgene were variably stable and showed differences in the rescue of dorsoventral cuticle defects caused by loss of *D-raf* maternal and zygotic function. These data provide evidence for the hypothesis that *D-raf* plays a role in two distinct signaling pathways that direct maturation of the embryonic ectoderm, the *Egfr* pathway for ventral cell determination and a second novel pathway required for the specification of lateral cell fates.

MATERIALS AND METHODS

Stocks, production of *D-raf* germline mosaics, and transgenic *D-raf* lines: *Egfr* embryos were collected from parents heterozygous for the top^{1P02}allele (Clifford and Schüpbach 1992) and the *CyO*, β*1* balancer chromosome that carried a *lacZ* gene under the control of the *fushi-tarazu* (*ftz*) promoter. To distinguish between wild-type, heterozygous, and homozygous *Egfr* embryos, the genotypes of the embryos were determined by following the expression pattern of the *lacZ* gene (see below). Embryos lacking the *lacZ* marker were homozygous lethal for the top^{1P02} allele. To generate mosaic females that were homozygous for the $D\text{-}raf^{11\text{-}29}$ protein null allele (Ambrosio *et al.* 1989a; Melnick *et al.* 1993) germline clones were made using the "FLP-DFS" technique of Chou and Perrimon (1992). Both *D-raf* "null" ($D\text{-}raf^{-}/Y$) and paternally rescued *D-raf* "torso" ($D\text{-}raf^{-}/+$) progeny, derived from eggs that lacked maternal D-raf protein, suffered embryonic lethality (Perrimon *et al.* 1985; Ambrosio *et al.* 1989b). To distinguish between these two embryo classes, females with *D-raf* germline clones were crossed with males of the genotype *yw / Y; P[w*$^+$ *ftz-β-gal G2] / P[w*$^+$ *ftz-β-gal G2]*. Embryonic genotypes were determined by following the expression pattern of the *lacZ* gene (see below). Embryos without the *lacZ* marker were referred to as *D-raf* null embryos because they lacked both maternal and zygotic D-raf protein and produced a small cuticular patch at the end of embryonic development. Their siblings, which expressed the *lacZ* gene, were referred to as paternally rescued *D-raf* torso embryos because they lacked only maternal *D-raf* gene activity and showed a terminal class phenotype in cuticular preparations.

Transgenic lines with modified *D-raf* genes were generated using standard *P*-element transformation (Spradling and Rubin 1982). Modified *D-raf* cDNAs as described in Baek *et al.* (1996) were inserted into the polylinker site of the pCaSpeR-hs vector (Thummel and Pirrotta 1992) using *Eco*RI and *Xba*I. Females with $D\text{-}raf^{11\text{-}29}$ germline clones were crossed to *yw / Y;* $P[w^+\ D\text{-}raf^{modified}]$ / $P[w^+\ D\text{-}raf^{modified}]$ transgenic males. In the case of the $D\text{-}raf^{S743A}$ gene, transgenic males were heterozygous for the $D\text{-}raf^{S743A}$ insertion on the *TM2* balancer chromosome. Thus, only one-half of the *D-raf* embryos inherited the $D\text{-}raf^{S743A}$ gene. This was considered in Western blots to determine D-raf^{S743A} protein concentration and in the phenotypic analysis of embryonic expression and cuticular patterns.

Rescue of *Egfr* embryos by central mRNA microinjection: $D\text{-}raf^{WT}$ (1 μg/μl) and $D\text{-}raf^{tor4021}$ (0.5 μg/μl) mRNA injections were as described in Baek and Ambrosio (1994) and Baek *et al.* (1996) with the following modifications: embryos from $top^{1P02}/+$ parents were collected for 30 min, aged 20 min (Nuclear Cycle 10), and then processed for central injection. We used chi-square analysis ($\chi^2 = \Sigma\ (O - E)^2/E$) and one degree of freedom and found that deviation from the expected genetic ratio of 3:1 was significant for those embryos injected with $D\text{-}raf^{tor4021}$ mRNA (P value of 0.90). As a control for the injection procedure, embryos from heterozygous *Egfr* parents were injected with $D\text{-}raf^{WT}$ mRNA. Deviation from the expected 3:1 ratio was not statistically significant in this case, with a P value of 0.90.

Phenotypic characterization of *Egfr*, *D-raf*, and transgenic *D-raf* embryos: *In situ* hybridizations were performed as described in Tautz and Pfeifle (1989). Antisense digoxigenin probes were prepared from plasmids containing the *otd* (Finkelstein and Perrimon 1990), *rho* (Bier *et al.* 1990), or *dpp* (Irish and Gelbart 1987) cDNAs. For double-labeling experiments to distinguish between wild-type, heterozygous, and homozygous *Egfr* embryos or *D-raf* torso and null embryos, antibodies were used at 1:1000 and 1:500 for the anti-β-gal primary (Sigma, St. Louis) and goat-anti-mouse secondary (Jackson ImmunoResearch, West Grove, PA) antibodies, respectively. Immunocytochemistry was performed as described in Perkins *et al.* (1996). For cuticular analysis, unhatched embryos were prepared according to Ashburner (1989). Embryos were photographed with a Zeiss Axioscope microscope using phase contrast or Nomarski optics.

In gene expression studies to measure the distance between *dpp* stripes, embryos were placed in 7% gelatin:63% glycerol mounting medium, were rolled onto their dorsal side, and then were covered with a coverslip. Measurements were performed on a Macintosh IIci computer using the public National Institutes of Health (NIH) Image program. Chi-square analysis (1 d.f. and a 95% confidence interval) showed that the separation between lateral *dpp* stripes was significantly different in homozygous *Egfr* and *D-raf* null embryos when compared with wild-type or *D-raf* torso embryos.

To assay for phenotypic rescue in *D-raf* null embryos by paternally inherited $D\text{-}raf^{modified}$ transgenes, embryos were collected for 1 hr, allowed to develop at 25° for 2.75 hr, and then heat-shocked at 37° for 0.5 hr. Embryos were transferred to 25° to continue development.

Western analysis: Western analysis was performed as described in Radke *et al.* (1997) with each sample containing 100 embryos. Eggs were collected over a 1-hr period from females with $D\text{-}raf^{11\text{-}29}$ germline clones after mating with *yw / Y;* $P[w^+\ D\text{-}raf^{modified}]$ / $P[w^+\ D\text{-}raf^{modified}]$ males. The embryos produced by these females were devoid of maternal D-raf protein. For non-heat-shocked samples, embryos were aged at 25° for 4.5 hr and then collected for processing, representing the 5-hr time point. For heat-shocked samples, embryos were aged for 3 hr and then heat-shocked for 30 min at 37°.

These embryos were allowed to recover for 1 or 6 hr before processing, representing the 5- and 10-hr samples, respectively. Since expression of each *D-raf*^{modified} gene was regulated by the *hsp70* promoter, the accumulation of 90-kD or truncated D-raf proteins was observed only after heat shock, with the exception of those lines with leaky transgenic expression. Densitomeric analysis was performed using the NIH Image program (developed at the National Institutes of Health and available from the internet by anonymous FTP from zippy.nimh.nih.gov or on floppy disk from the National Technical Information Service, Springfield, VA, part no. PB95-500195-GEI) with individual molecular weight contributions of the truncated D-raf proteins considered in this analysis.

RESULTS

***D-raf* acts downstream of *Egfr* for the establishment of ventral ectodermal cell fates:** In the Drosophila embryo, *Egfr* activity is required to instruct a field of cells that lie on either side of the ventral furrow to adopt a ventral ectodermal fate. It is from this neuroectodermal cell population that the ventral nervous system and epidermis arise. At later times, *Egfr* functions in germband retraction and cuticle formation. Embryos that develop without *Egfr* activity fail to form ventral cuticular structures and show the "faint little ball" phenotype (Figure 1B). We used a constitutively active form of the D-raf protein, D-raf^{tor4021}, to bypass the requirements for *Egfr* function in embryos that lacked *Egfr* gene activity. For the generation of hyperactive D-raf^{tor4021}-proteins, the extracellular and transmembrane domains of the *torso RTK* gene were fused to the *D-raf* kinase domain. Chimera D-raf^{tor4021}proteins were shown to act independently of *sevenless RTK* gene function in developing photoreceptor cells and exhibited gain-of-function effects in the *Tor* signaling pathway (Dickson and Hafen 1994; Baek *et al.* 1996).

We tested whether this activated D-raf protein would act independently of *Egfr* to rescue the embryonic lethality associated with homozygous mutations in the *Egfr* gene (Table 1). In the case of our noninjected control, 25% of the embryos derived from heterozygous *Egfr* parents (*Egfr*$^{-}$/+) failed to hatch, showed the faint little ball phenotype, and were homozygous for the *Egfr* mutation. We used *D-raf*^{WT} mRNA as a control for the injection procedure and found that after injection 27% of the embryos from heterozygous *Egfr* parents failed to hatch. These embryos showed the *Egfr* mutant phenotype at 24 hr. When *D-raf*^{tor4021} mRNA was injected into the central region of embryos collected from heterozygous *Egfr* parents, all aspects of defective *Egfr* signaling were rescued for some of the mutant *Egfr* embryos. Of the 258 embryos that received injection, 217 (84%) hatched out of their egg cases as larvae, while 41 (16%) remained within their eggshells. Thus, we observed an increase in embryonic hatching and suppression of *Egfr*-induced lethality after injection of *D-raf*^{tor4021} mRNA. We calculated and found a statistically significant difference in hatching rate for embryos that had received *D-raf*^{tor4021} mRNAs when compared to those that did not (see materials and methods). We also found partial rescue of the *Egfr* phenotype in unhatched embryos that had received *D-raf*^{tor4021} mRNAs with ventral cuticular structures observed (Figure 1C). We concluded that constitutively active D-raf^{tor4021} molecules could bypass the requirement for *Egfr* activity in the embryo and direct cells of the embryonic ectoderm to adopt a ventral fate. These results showed that *D-raf* participates downstream of *Egfr* in developing embryos.

Specification of ectodermal cell fates in *D-raf* null embryos: Once we had found that an activated form of the D-raf protein could suppress the effects of a loss-of-function *Egfr* allele, we reasoned that embryos lacking maternal and zygotic *D-raf* activity would exhibit an *Egfr*-like phenotype. We expected that these embryos would also show defects associated with the loss of maternal *D-raf* function in *Tor* signaling. To determine whether the identities of cells in the ventral ectoderm were dependent on *D-raf* activity, marker gene expression patterns and cuticles produced by *D-raf* embryos were compared to those of wild-type and *Egfr* embryos. To generate these *D-raf* embryos, mosaic *D-raf* females were produced whose eggs lacked maternal D-raf proteins (see materials and methods). Once fertilized, these eggs gave rise to two classes of embryos (Perrimon *et al.* 1985; Ambrosio *et al.* 1989b): the first class was composed of the paternally rescued *D-raf* torso embryos (*D-raf*$^{-}$/+) that had inherited a wild-type *D-raf* gene from their fathers, were defective in *Tor* RTK signaling and were missing head and tail structures at 24 hr (Figure 1D). These *D-raf* torso embryos lacked maternal but not zygotic *D-raf* activity. The second phenotypic class was composed of the *D-raf* null embryos (*D-raf*$^{-}$/*Y*) whose exoskeletons consisted of what appeared to be a small patch of dorsal cuticle (Figure 1E). These embryos lacked maternal and zygotic *D-raf* activity throughout development. We anticipated that this *D-raf* null embryonic class would exhibit the phenotypic characteristics consistent with defective *Egfr* signaling, a consequence of defective D-raf protein activity.

First, to determine whether the establishment of ventral cell identity by the maternal dorsal gene system occurred normally in *D-raf* embryos we assayed the accumulation of *rhomboid* (*rho)* mRNAs between 4 and 6 hr (stages 9–12) of development (Bier *et al.* 1990). As visualized by *in situ* hybridization, a column of cells ~2–3 wide on either side of the ventral midline showed the accumulation of *rho* mRNAs (data not shown). This temporal and spatial pattern of *rho* expression was observed in all embryos in our *D-raf* collections with each embryo a member of either the *D-raf* torso or null class. An equivalent *rho* expression pattern was observed in wild-type and *Egfr* embryos. Thus, the initial step in the establishment of ventral cell identity, by *dorsal* and other maternal genes that act to define the dorsoventral embryonic axis, was not perturbed when these events

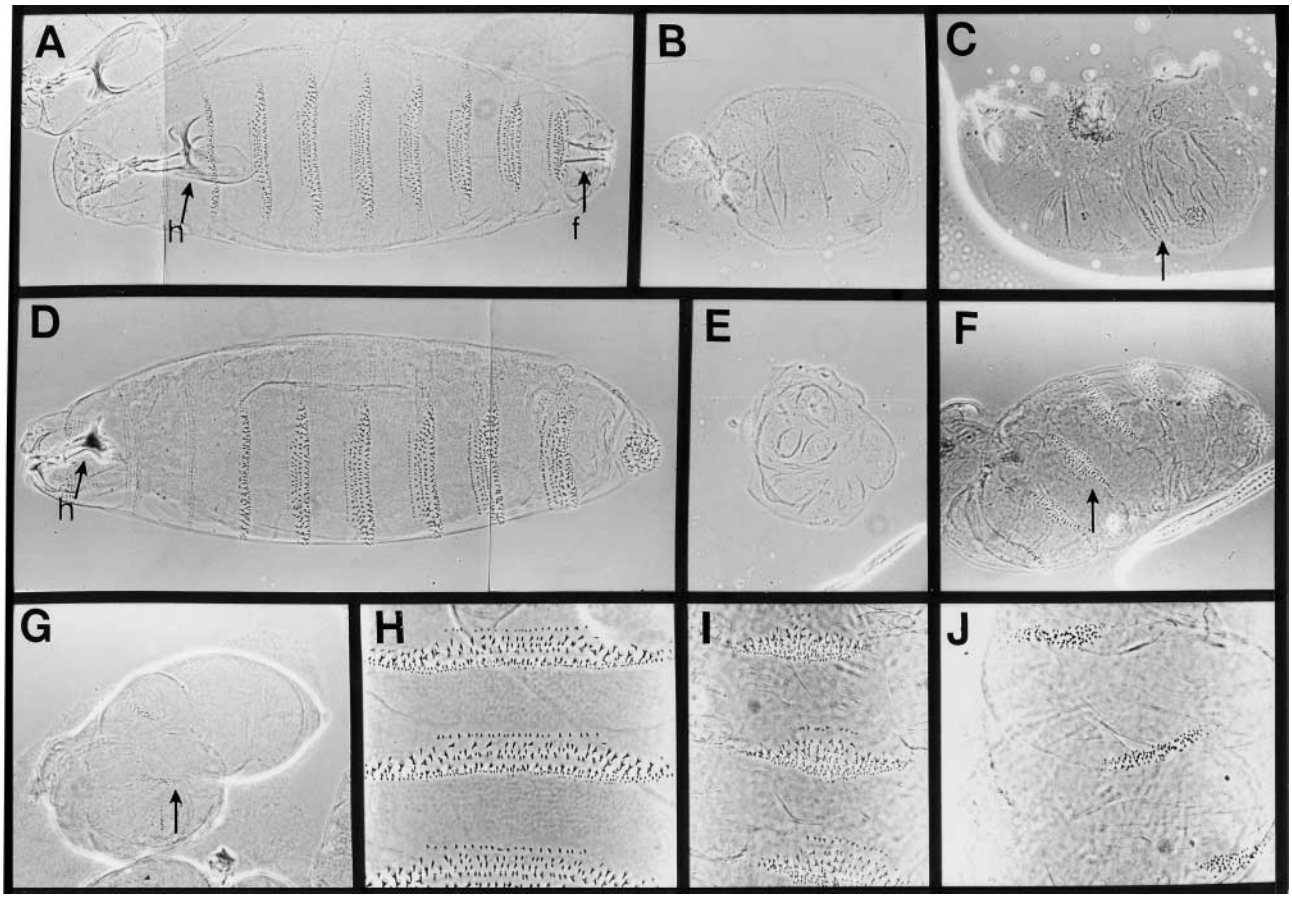

FIGURE 1.—Cuticular preparations of *Egfr* and *D-raf* mutant embryos. (A) A wild-type embryo with inverted head skeleton (h), posterior filzkörper (f), and ventral abdominal denticle bands. (B) View of an *Egfr* homozygote devoid of denticle setae and lacking anterior, posterior, and ventral cuticular structures. (C) Partial rescue of an *Egfr* homozygote after central injection with *D-raf*^{tor4021} mRNA. There was some restoration of cuticular structures, including ventral denticles (arrow). For *D-raf* embryos that developed without maternal *D-raf* activity two phenotypes were observed: (D) A *D-raf* torso embryo with a truncated head skeleton (h), seven abdominal denticle bands, and missing tail structures and (E) a *D-raf* null embryo that produced a small patch of cuticle with few distinguishing features. Expression of transgenic D-raf proteins often resulted in partial rescue of the *D-raf* null phenotype with embryos showing either the "imperfect torso" or "null with denticles" phenotype. (F) A representative of the "imperfect torso" embryonic class with robust cuticle and denticle bands approximately two-thirds the width of a wild-type band. (G) A representative of the "null with denticles" embryonic class has faint cuticle and denticle belts approximately one-third the width of a wild-type band. Typically each denticle band consisted of four or five rows of shortened setae similar to those setae that lie at the lateral edges of denticle belts from wild-type embryos indicative of a deletion in the central most pattern with a corresponding expansion of more ventral lateral elements. High-power-magnification views of denticle belts from (H) wild-type, (I) "imperfect torso," and (J) "null with denticles" embryos.

took place in the absence of maternal or zygotic *D-raf* activity.

To determine whether *EGR-receptor* signaling occurred normally in *D-raf* embryos, expression of the *orthodenticle (otd)* gene was monitored (RAZ and SHILO 1993). In wild-type control embryos, at 6 hr (stage 11) *otd* mRNAs

TABLE 1

Rescue of *Egfr* mutant embryos by central injection of *D-raf*^{tor4021} mRNA

mRNA	No. of embryos from heterozygous *Egfr* parents assayed	No. of unhatched embryos (% total)	No. of hatched embryos (% total)
Noninjected	100	25 (25)	75 (75)
D-raf^{WT}	154	42 (27)	112 (73)
D-raf^{tor4021}	258	41 (16)	217 (84)

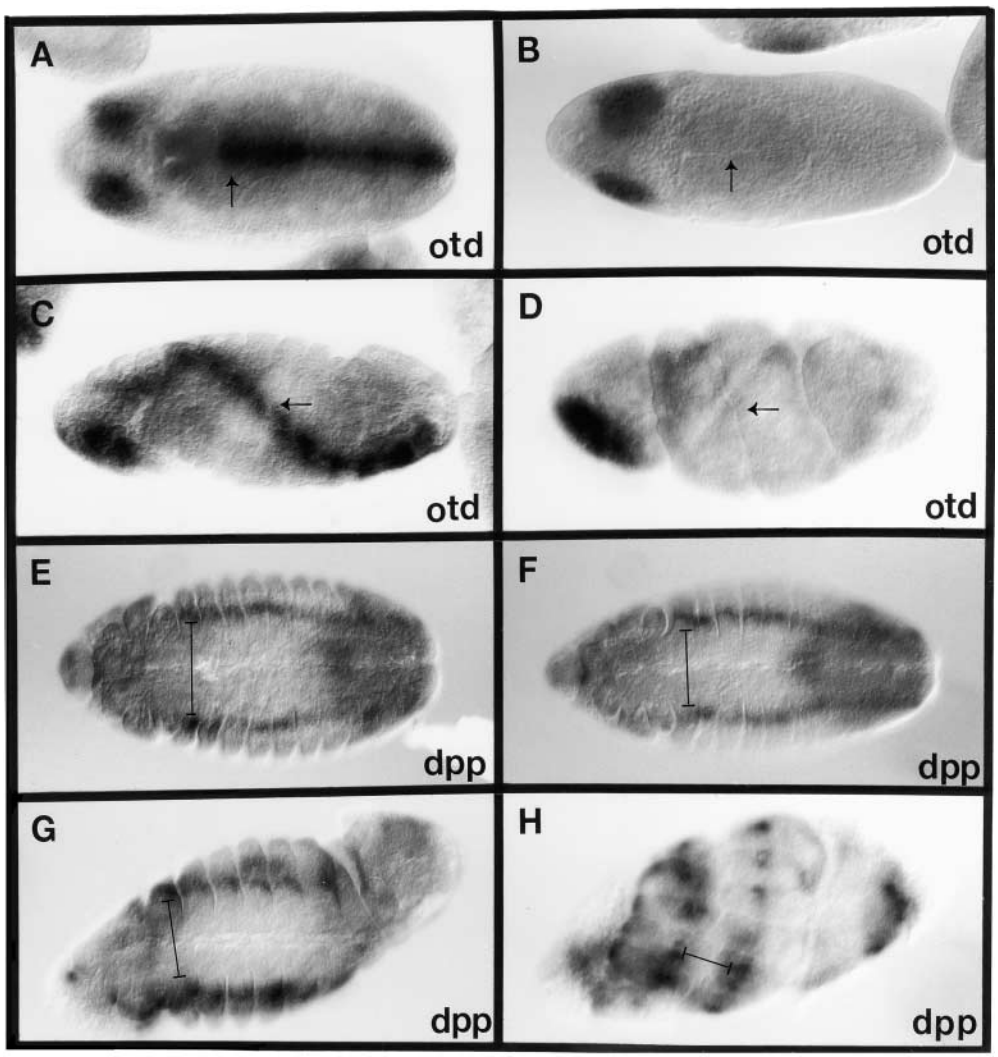

FIGURE 2.—The accumulation of *otd* mRNAs at 6 hr and *dpp* mRNAs at 10 hr in *Egfr* and *D-raf* embryos. (A) *otd* mRNAs accumulated in two head placodes and a ventral midline stripe (arrow) in wild-type and heterozygous *Egfr* embryos. (B) Homozygous *Egfr* embryos lacked expression of *otd* mRNA along the ventral midline. (C) *D-raf* torso embryos showed head and ventral midline accumulation of *otd* mRNAs and were twisted. (D) Twisted *D-raf* null embryos lacked ventral *otd* stripe expression. (E) At 10 hr the accumulation of *dpp* mRNA was in two lateral stripes for wild-type and heterozygous *Egfr* embryos. (F) Homozygous *Egfr* embryos showed a reduced distance between lateral *dpp* stripes. (G) *D-raf* torso embryos had a wild-type pattern of *dpp* mRNA accumulation. (H) *D-raf* null embryos showed little separation between lateral *dpp* stripes and were twisted.

accumulated in cells adjacent to the ventral midline and in the head (Figure 2A). In embryos lacking *Egfr* activity, *otd* expression occurred only in those cells within the embryonic head (Figure 2B). In *D-raf* embryo collections, we observed two patterns of embryo staining with approximately one-half of the embryos showing *otd* expression in cells along the ventral midline and in the head (Figure 2C). For the remaining *D-raf* embryos, the accumulation of *otd* mRNAs was observed only in the head, similar to *Egfr* embryos (Figure 2D).

To distinguish between torso and null embryos in our *D-raf* collections, we used a *ftz*-β-*gal* marker gene located on the paternal *X* chromosome. Males with the *ftz*-β-*gal* gene were allowed to fertilize eggs from mosaic females that lacked *D-raf* activity (data not shown). In this double-labeling experiment, embryos that showed a *ftz* pattern of β-*gal* expression were assigned to the *D-raf* torso class. These embryos also displayed a wild-type pattern of *otd* expression. In those *D-raf* null embryos lacking β-*gal* expression, *otd* mRNAs were detected only in cells of the head, similar to *Egfr* embryos.

As was shown previously, between 4 and 7 hr (stages 9–11) of development wild-type and *Egfr* embryos accumulated *decapentaplegic* (*dpp*) mRNAs in cells that formed two lateral stripes, when embryos were viewed ventrally (CLIFFORD and SCHÜPBACH 1992; RAZ and SHILO 1993). We observed a similar pattern of *dpp* mRNA accumulation in *D-raf* mutant embryos at this developmental stage (data not shown). However, CLIFFORD and SCHÜPBACH (1992) showed that the ventral distance between *dpp* stripes becomes smaller in *Egfr* embryos as they develop. We recorded and compared the distance between lateral *dpp* stripes in wild-type, *Egfr*, and *D-raf* embryos at 10 hr (stage 13) of development (Table 2). For wild-type embryos the average stripe distance was 0.111 units. In our collection of *Egfr* embryos, ~75% showed an average *dpp* lateral stripe distance of 0.118 units, similar to wild type (Figure 2E). This phenotypic class contained embryos that were heterozygous mutant ($Egfr^{-}$/+) or wild type with respect to the *Egfr* gene. In the remaining 25% of the embryos the average *dpp* stripe distance was reduced to 0.075 units as anticipated for homozygous mutant *Egfr* embryos (Figure 2F).

Two phenotypic classes of *D-raf* embryos were also

TABLE 2

Average distance between lateral *dpp* stripes in the third thoracic segment of embryos at 10 hr (stage 13) of development measured in arbitrary units

Embryos	(*n*)	Stripe distance
Wild type	22	0.111 ± 0.01
$Egfr^{-\ or\ +}/Egfr^{+}$	62	0.118 ± 0.01
$Egfr^{-}/Egfr^{-}$	19	0.075 ± 0.03
$D\text{-}raf^{+}/D\text{-}raf^{-}$	22	0.120 ± 0.01
$D\text{-}raf^{-}/Y$	22	0.064 ± 0.01

distinguished on the basis of a statistically relevant difference in *dpp* stripe distance (Figure 2, G and H). In approximately one-half of the embryos the average *dpp* lateral stripe distance was 0.120 units, with the remaining embryos showing an average separation of 0.064 units (Table 2). We speculated that this second phenotypic class contained the *D-raf* null embryos (see MATERIALS AND METHODS). To test this idea, the marker *ftz*-β-*gal X* chromosome was again employed in a double-labeling experiment to distinguish between *D-raf* torso and null embryos (data not shown). As anticipated, it was the male *D-raf* null embryonic class that showed the decrease in distance between lateral *dpp* stripes, indicative of a loss in ventral cell fates.

On the basis of our analysis of *rho*, *otd*, and *dpp* gene expression patterns in *D-raf* null embryos, we concluded that ventral ectoderm cells were specified incorrectly in the absence of *D-raf* activity. This loss resulted in the production of a mature *D-raf* null exoskeleton that was severely reduced in size and devoid of ventral structures, consistent with the *Egfr* embryonic phenotype. However, when we compared the distance between lateral *dpp* stripes in *Egfr* (0.075 units) and *D-raf* null (0.064 units) embryos, it was smaller in *D-raf* null embryos. In addition, after cursory inspection, the size of the exoskeleton patch produced by *D-raf* null embryos appeared smaller than that from *Egfr* embryos (Figure 1, B and E). We speculated that these differences could be biologically significant and expanded our analysis to address this potentially interesting finding.

The role of *D-raf* in the embryonic ectoderm: To better understand the role that *D-raf* plays in the ectoderm and to access its regulation in various developmental pathways we utilized partial loss-of-function alleles of *D-raf* generated *in vitro* (BAEK *et al.* 1996). *D-raf* shares homology with family members in CR1 that contains *D-ras* binding motifs; CR2, a region rich in serine and threonine residues; and the CR3 kinase domain (Figure 3A). CR1 is thought to exhibit positive control in the regulation of the D-raf protein via its interaction with *D-Ras*, while CR2 appears to be involved in the negative regulation of the molecule (HOU *et al.* 1995; BAEK *et al.* 1996). We tested whether conserved subdomains, CR1 and CR2, or putative phosphorylation sites, serine 388 or 743, were essential for the activity of *D-raf* in the embryo or involved in its positive or negative regulation.

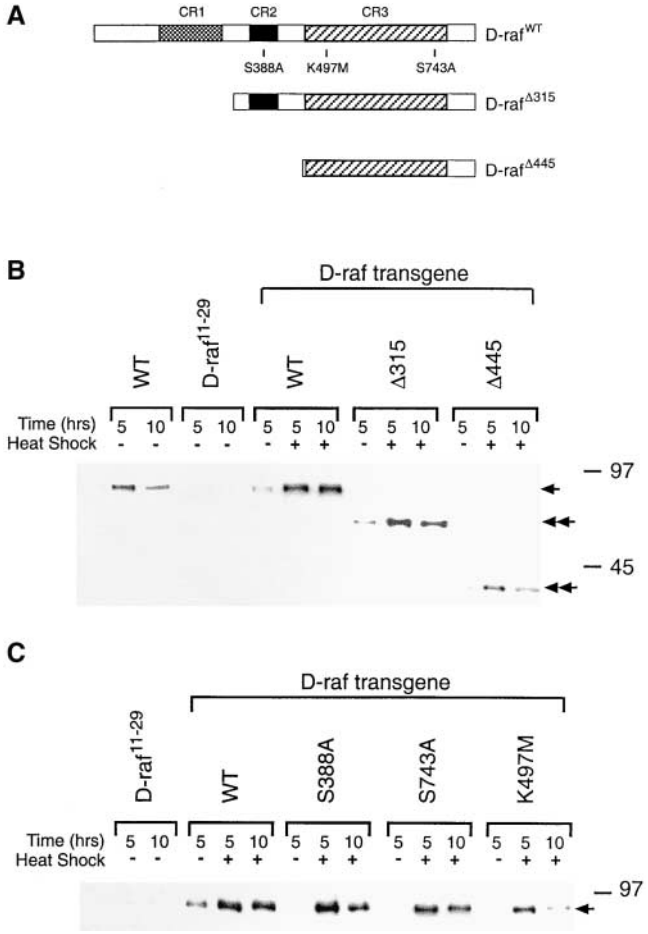

FIGURE 3.—A linear representation of D-raf proteins expressed in *D-raf* embryos and their accumulation as visualized by Western analysis. (A) The wild-type D-raf protein shows three regions of homology with *Raf* family members: conserved region 1 (CR1), CR2, and CR3. $D\text{-raf}^{K497M}$ proteins contained the amino acid substitution, lysine to methionine, at residue 497. Amino acid substitutions to alanine were also generated at serine 388 ($D\text{-}raf^{S388A}$) and serine 743 ($D\text{-}raf^{S743A}$). The $D\text{-raf}^{\Delta 315}$ protein was missing the first 315 amino acids of *D-raf*, while the $D\text{-raf}^{\Delta 445}$ protein lacked residues 1–445. (B) Accumulation of wild-type and $D\text{-raf}^{modified}$ protein in embryos at 5 and 10 hr of development, with (+) or without (−) heat shock. The *hsp 70* promoter was used to regulate $D\text{-}raf^{modified}$ gene expression and controlled by heat shock. The first two sets of lanes show the accumulation of D-raf proteins from wild-type (WT) and $D\text{-}raf^{11\text{-}29}$ embryos that lacked maternal D-raf proteins. In $D\text{-}raf^{11\text{-}29}$ embryo collections only embryos of the *D-raf* torso class produce zygotic D-raf protein. The accumulation of this zygotic D-raf protein was very low with almost none detected here. For the *D-raf* transgenic lanes with embryos derived from homozygous $D\text{-}raf^{11\text{-}29}$ germlines the accumulation of 90-kD $D\text{-raf}^{WT}$, 60-kD $D\text{-raf}^{\Delta 445}$, and 38-kD $D\text{-raf}^{\Delta 315}$ proteins was detected. (C) Accumulation of D-raf proteins with amino acid substitutions at 5 and 10 hr of embryonic development with (+) and without (−) heat shock. High levels of these 90-kD D-raf proteins accumulate at 5 hr after heat induction.

These modifications of *D-raf* often resulted in decreased *D-raf* activity. Thus, by expressing partial loss-of-function *D-raf* alleles in *D-raf* null embryos we were successful in deciphering the role *D-raf* plays in developing embryos.

Using a structure-function strategy, we generated several modified forms of the D-raf protein (Figure 3A). The $D\text{-}raf^{WT}$ and $D\text{-}raf^{K497M}$ genes were constructed as positive and negative controls, respectively, with the $D\text{-}raf^{WT}$ allele a full-length copy of a *D-raf* cDNA (Sprenger *et al.* 1993). $D\text{-}raf^{K497M}$ lysine 497, which was shown to be critical for D-raf protein kinase activity and likely involved in ATP binding, was replaced with a methionine (Sprenger *et al.* 1993; Baek *et al.* 1996). The N-terminal and CR1 deletion mutation, $D\text{-}raf^{\Delta 315}$, was likely to show a partial loss-of-function in *D-raf* null embryos. For the $D\text{-}raf^{\Delta 445}$ mutation both positive (CR1) and negative (CR2) control elements were lost, and we predicted that this form of *D-raf* would act in a manner similar to wild type or, on the basis of its structural similarity to oncogenic forms of *Raf-1*, show a gain-of-function effect in the embryo. Of the five phosphorylation sites identified for the human *Raf-1* kinase, two were conserved in the D-raf protein (Morrison *et al.* 1993). Baek *et al.* (1996) generated serine to alanine substitutions at these sites and showed that S388 (CR2) played a negative role while S743 (CR3) was involved in the positive control of *D-raf* in the *Tor* pathway. We predicted that the $\text{D-raf}^{\text{S388A}}$ and $\text{D-raf}^{\text{S743A}}$ proteins would show similar phenotypic consequences for developing cells in the embryo.

Using *P*-element-mediated transformation, we generated Drosophila lines that contained an insertion of the $D\text{-}raf^{WT}$, $D\text{-}raf^{K497M}$, $D\text{-}raf^{\Delta 315}$, $D\text{-}raf^{\Delta 445}$, $D\text{-}raf^{S388A}$, or $D\text{-}raf^{S743A}$ gene on either the second or third chromosome. Each of these modified *D-raf* genes were paternally introduced into *D-raf* embryos lacking maternal D-raf protein (see materials and methods). We also monitored the level and stability of D-raf proteins produced by expression of each paternally inherited $D\text{-}raf^{modified}$ gene. In this assay 100 embryos were collected for each sample and processed for Western analysis (Figure 3, B and C). Since the expression of each $D\text{-}raf^{modified}$ gene was under the control of the *hsp70* promoter, samples were processed from non-heat-shocked or heat-shocked embryos at 5 and 10 hr of development. We found that these $\text{D-raf}^{\text{modified}}$ proteins were variably stable and in *D-raf* null embryos showed differences in the rescue of dorsoventral cuticular defects caused by the loss of *D-raf* maternal and zygotic function. We organized our results on the basis of degree of phenotypic rescue that was observed in *D-raf* null embryos with the activity of $D\text{-}raf^{WT} > D\text{-}raf^{S388A} > D\text{-}raf^{\Delta 445} > D\text{-}raf^{S743A} > D\text{-}raf^{\Delta 315} > D\text{-}raf^{K497M}$ and these findings are presented below.

Rescue of the D-raf null phenotype by paternally inherited $D\text{-}raf^{WT}$ proteins: We assayed the accumulation of D-raf protein in *D-raf* embryos that had inherited the $D\text{-}raf^{WT}$ gene. For these embryos the accumulation of D-raf proteins after heat induction was approximately twofold greater than that found in wild-type embryos at 5 hr (Figure 3B). At 10 hr, the level of the D-raf^{WT} protein was unchanged. We also determined the effect of D-raf^{WT} proteins on *otd* and *dpp* gene expression patterns in *D-raf* embryos. As anticipated, induction of the $D\text{-}raf^{WT}$ gene resulted in 100% of the *D-raf* null class showing wild-type ventral *otd* stripe expression and a normal pattern of *dpp* expression (Table 3). We also examined embryonic cuticles at 24 hr to assess the ability of the $D\text{-}raf^{WT}$ gene to promote signaling in the late-stage *Egfr* pathway responsible for epidermal differentiation and the final cuticular pattern (Clifford and Schüpbach 1992; Raz and Shilo 1993). Of these *D-raf* null embryos that had inherited the $D\text{-}raf^{WT}$ gene, 99% developed cuticles indistinguishable from their *D-raf* torso sisters (Table 4). Thus, all ectodermal signaling pathways dependent on *D-raf* activity could be fully restored in null embryos by expression of the $D\text{-}raf^{WT}$ gene.

The consequence of $D\text{-}raf^{S388A}$ and $D\text{-}raf^{\Delta 445}$ expression in D-raf null embryos: Figure 3C shows the quantity of serine to alanine substituted D-raf proteins generated by the $D\text{-}raf^{S388A}$ gene. When compared with the expression of the $D\text{-}raf^{WT}$ gene at 5 hr, an equivalent accumulation of $\text{D-raf}^{\text{S388A}}$ protein was observed, with a slight reduction at 10 hr. In our phenotypic analysis, 84% of these $D\text{-}raf^{S388A}$ expressing *D-raf* null embryos showed rescue of *Egfr*-induced *otd* expression in ventral cells and the distance between *dpp* stripes appeared normal (Table 3). By the completion of embryonic development, 97% of the *D-raf* null embryos showed the torso phenotype, while the remaining 3% showed a composite "imperfect torso" phenotype (Table 4). In addition to showing head and tail defects associated with the torso phenotype, embryos of the "imperfect torso" class were twisted and had denticle bands of reduced width, indicative of partial loss of signaling in ventral cells that depend on the *Egfr* pathway for development (Figure 1, F and I). Since all of the *D-raf* null embryos showed some phenotypic rescue by $D\text{-}raf^{S388A}$, we concluded that serine 388 was not essential for the function of *D-raf* in the ectoderm. Instead it was likely that S388 plays a negative role in the regulation of *D-raf* similar to its function in *Tor* signaling (Baek *et al.* 1996).

For *D-raf* null embryos that inherited the $D\text{-}raf^{\Delta 445}$ gene, 52% showed rescue of the *Egfr*-induced *otd* expression pattern (Table 3). This was approximately one-half the percentage rescued by the $D\text{-}raf^{WT}$gene, although the quantity of truncated ~38-kD D-raf protein in these embryos was equivalent to that observed for *D-raf* embryos expressing the $D\text{-}raf^{WT}$ gene at 5 hr (Figure 3B). For the human Raf-1 protein, removal of CR1 and CR2 resulted in unregulated kinase activity (Bonner *et al.* 1985; Rapp *et al.* 1988). We assessed whether the $\text{D-raf}^{\Delta 445}$ protein acted ectopically to create a wide ventral *otd* stripe, but all of the *otd* stripes were of wild-type width (data not shown). When *dpp* mRNA patterns were

TABLE 3

***otd* and *dpp* expression in *D-raf* null embryos with transgenic D-raf proteins**

Transgene	No. of *D-raf* null embryos with transgene[a]	Rescue of *otd* stripe expression: *D-raf* null embryos with transgene[a] that show an *otd* ventral stripe (% of total)	Average distance between lateral *dpp* stripes in the third thoracic segment at 10 hr for *D-raf* null embryos with transgene[a] in arbitrary units ($n = 5$)
$D\text{-}raf^{WT}$	132	132 (100)	0.111 ± 0.01
$D\text{-}raf^{S388A}$	187	157 (84)	0.116 ± 0.02
$D\text{-}raf^{\Delta 445}$	219	113 (52)	0.113 ± 0.02
$D\text{-}raf^{S743A}$	34	0 (0)	0.089 ± 0.02
$D\text{-}raf^{\Delta 315}$	129	0 (0)	0.063 ± 0.01
$D\text{-}raf^{K497M}$	210	0 (0)	0.047 ± 0.03

[a] *D-raf* torso embryos from *D-raf* mutant germlines show *otd* ventral stripe expression. The expected *D-raf* null embryos referred to here do not express the ventral *otd* stripe in the absence of a *D-raf* transgene.

analyzed in $D\text{-}raf^{\Delta 445}$ expressing null embryos the distance between lateral stripes in the third thoracic segment at 10 hr was similar to those that had inherited the $D\text{-}raf^{WT}$ gene (Table 3).

In the analysis of 24-hr cuticular patterns 52% of the $D\text{-}raf^{\Delta 445}$ embryos were rescued and showed the torso phenotype (Table 4). For the remaining embryos, partial rescue was observed with signaling by the D-raf$^{\Delta 445}$ protein defective in the determination of the ventral ectoderm. Of these embryos, 18% showed the "imperfect torso" phenotype and 30% showed the "null with denticles" phenotype (Figure 1, G and J). These "null with denticles" embryos were twisted, had faint cuticles with narrow denticle bands, and were phenotypically similar to *Egfr* embryos homozygous for intermediate defective alleles of *Egfr* (Clifford and Schüpbach 1992; Raz and Shilo 1992, 1993). Overall, we found that signal transmission by $D\text{-}raf^{\Delta 445}$ was less reliable when compared with $D\text{-}raf^{WT}$, although the D-raf$^{\Delta 445}$ protein had the potential to rescue all aspects of the embryonic *D-raf* null phenotype.

The consequence of $D\text{-}raf^{S743A}$ and $D\text{-}raf^{\Delta 315}$ expression in D-raf null embryos: Analysis of *D-raf* embryos expressing the $D\text{-}raf^{S743A}$ gene was somewhat complicated by the insertion of $D\text{-}raf^{S743A}$ on the *TM2* balancer chromosome. Thus, only one-half of the *D-raf* null embryos fertilized by $D\text{-}raf^{S743A}$ transgenic males inherited the $D\text{-}raf^{S743A}$ gene. We determined the amount of D-raf^{S743A} protein that accumulated in *D-raf* embryos with the $D\text{-}raf^{S743A}$ gene and found that it was ~1.5-fold greater than that observed for those embryos that had inherited the $D\text{-}raf^{WT}$ gene (Figure 3C). Although greater levels of this modified D-raf protein accumulated in *D-raf* null embryos expressing the $D\text{-}raf^{S743A}$ gene, *otd* stripe expression was not observed (Table 3). Also, the distance between lateral *dpp* stripes in these $D\text{-}raf^{S743A}$ embryos was diminished when compared with wild type, but not to the degree observed for embryos expressing the $D\text{-}raf^{\Delta 315}$ or $D\text{-}raf^{K497M}$ genes, as presented below. Thus, the specification of ventral cell fates at the midline requires the positive regulation of the D-raf protein at serine 743.

Accordingly, 99% of the *D-raf* null embryos expressing the $D\text{-}raf^{S743A}$ gene showed the "imperfect torso" phenotype (Table 4). To better assess the pattern deletions

TABLE 4

Cuticle formation in *D-raf* null embryos with transgenic D-raf proteins

Transgene	*D-raf* null embryos with transgene[a]	Phenotypic classification of *D-raf* null embryos: Torso embryos (% of total)	Imperfect torso embryos (% of total)	Null with denticle embryos (% of total)	Null embryos (% of total)
$D\text{-}raf^{WT}$	96	95 (99)	0 (0)	0 (0)	1 (1)
$D\text{-}raf^{S388A}$	62	60 (97)	2 (3)	0 (0)	0 (0)
$D\text{-}raf^{\Delta 445}$	50	26 (52)	9 (18)	15 (30)	0 (0)
$D\text{-}raf^{S743A}$	98	0 (0)	97 (99)	0 (0)	1 (1)
$D\text{-}raf^{\Delta 315}$	102	3 (1)	0 (0)	83 (81)	16 (16)
$D\text{-}raf^{K497M}$	185	0 (0)	0 (0)	0 (0)	185 (100)

[a] *D-raf* torso embryos from *D-raf* mutant germlines show *otd* ventral stripe expression. The expected *D-raf* null embryos referred to here do not express the ventral *otd* stripe in the absence of a *D-raf* transgene.

TABLE 5

Cuticular analysis of *D-raf* null embryos with transgenic D-raf proteins in arbitrary units ($n = 5$)

Transgene	Width of second abdominal denticle band	Third thoracic segment		
		Distance between Keilin's organs	Distance between ventral black dots	Distance between ventral and dorsal black dots
D-raf^{WT}	0.284 ± 0.04	0.140 ± 0.01	0.248 ± 0.04	0.136 ± 0.01
D-raf^{S388A}	0.304 ± 0.02	0.139 ± 0.03	0.242 ± 0.02	0.135 ± 0.03
D-raf$^{\Delta 445}$	0.280 ± 0.03	0.138 ± 0.02	0.237 ± 0.02	0.132 ± 0.02
D-raf^{S743A}	0.179 ± 0.01	0.082 ± 0.01	0.160 ± 0.04	0.090 ± 0.01
D-raf$^{\Delta 315}$	0.106 ± 0.04	Absent	0.050 ± 0.01	0.078 ± 0.01
D-raf^{K497M}	Absent	Absent	Absent	Absent

generated by the loss of epidermal cell fates in these *D-raf*^{S743A} embryos we scored epidermal sensory organs that develop in ventral and lateral domains of the embryo. The separation between Keilin's organs and ventral black dots on the ventral surface was measured. Also, to determine whether patterning in lateral cells was normal for these embryos the distance between ventral and dorsal black dots was recorded. When compared with wild type, *D-raf*^{S743A} embryonic cuticles showed a decreased distance between Keilin's organs and ventral black dots (Table 5 and Figure 4B). A decrease in the distance between ventral and dorsal black dot material was also observed (Figure 4E). This later finding proved very informative for it led to the hypothesis that a novel pathway, dependent upon the D-raf protein, was operating for signal transmission in cells undergoing lateral epidermal development. It appears that cell fate specification in the ventralmost ectoderm via the EGR receptor and proper development of a subpopulation of lateral cells requires an optimal level of *D-raf* activity that was not achieved by the D-raf^{S743A} protein.

Rescue of epidermal patterning defects was further diminished in *D-raf* null embryos that expressed the *D-raf*$^{\Delta 315}$ gene. Using Western analysis we found that the D-raf$^{\Delta 315}$ protein migrated as an ~60-kD band detected at a level equivalent to that of the 90-kD D-raf^{WT} protein at 5 hr (Figure 3B). Approximately 80% of this D-raf$^{\Delta 315}$ protein was present at 10 hr. When *D-raf* null embryos that inherited the *D-raf*$^{\Delta 315}$ gene were assayed for *otd* and *dpp* stripe expression, ventral *otd* expression was not observed and the distance between lateral *dpp* stripes was much reduced when compared with embryos expressing the *D-raf*^{WT} gene (Table 3). Thus, a substantial decrease in the output of the *Egfr*-induced signal was detected. By the completion of development, 83 (81%) of the expected 102 *D-raf* null embryos with D-raf$^{\Delta 315}$ protein showed cuticles with the "null with denticles" phenotype (Table 4).

We also identified epidermal sensory organs in *D-raf* null embryos expressing the *D-raf*$^{\Delta 315}$ gene and made note of their relative positions (Table 5). Significantly, an absence of Keilin's organs was recorded and a corresponding expansion in the size of ventral black dot material was observed (Figure 4C). The distance between these enlarged ventral dots was substantially reduced when compared with wild-type embryos (Table 5). A reduction in the distance between ventral and dorsal black dot sensory organs was also observed (Figure 4, F–H). This finding again implicates *D-raf* in a pathway required for the development of lateral cells. Thus, by reducing the ability of the D-raf protein to act in signaling we have verified its role in the *Egfr* pathway and have also uncovered its function in a novel pathway involved in lateral cell development.

The consequence of D-raf^{K497M} *proteins in developing D-raf null embryos:* As anticipated, *D-raf*-dependent pathways were not rescued when *D-raf* null embryos expressed the kinase defective *D-raf*^{K497M} gene. For these embryos the accumulation of D-raf^{K497M} proteins after heat induction was ~2-fold greater than that found in *D-raf*^{WT} embryos at 5 hr (Figure 3C). However, by 10 hr this level was dramatically reduced ~0.75-fold, indicating that the K497M modification renders the D-raf protein unstable. Induction of the kinase defective *D-raf*^{K497M} gene did not restore wild-type *otd* or *dpp* expression patterns in *D-raf* null embryos (Table 3). When assessed after completion of embryogenesis, those embryos that had inherited the *D-raf*^{K497M} gene showed the *D-raf* null phenotype (Tables 4 and 5). Thus, the kinase activity of the D-raf protein proved essential in those embryonic cells that utilize *D-raf* for signal transmission.

DISCUSSION

The role of *D-raf* in embryonic dorsoventral patterning: Along the dorsoventral egg perimeter, embryonic cell fates are first established by the dorsal maternal patterning system (reviewed in Anderson 1998). Elaboration of this pattern in the ectoderm is dependent upon the Dpp protein in dorsal cells and the EGR receptor in ventral cells (Schweitzer and Shilo 1997; Podos

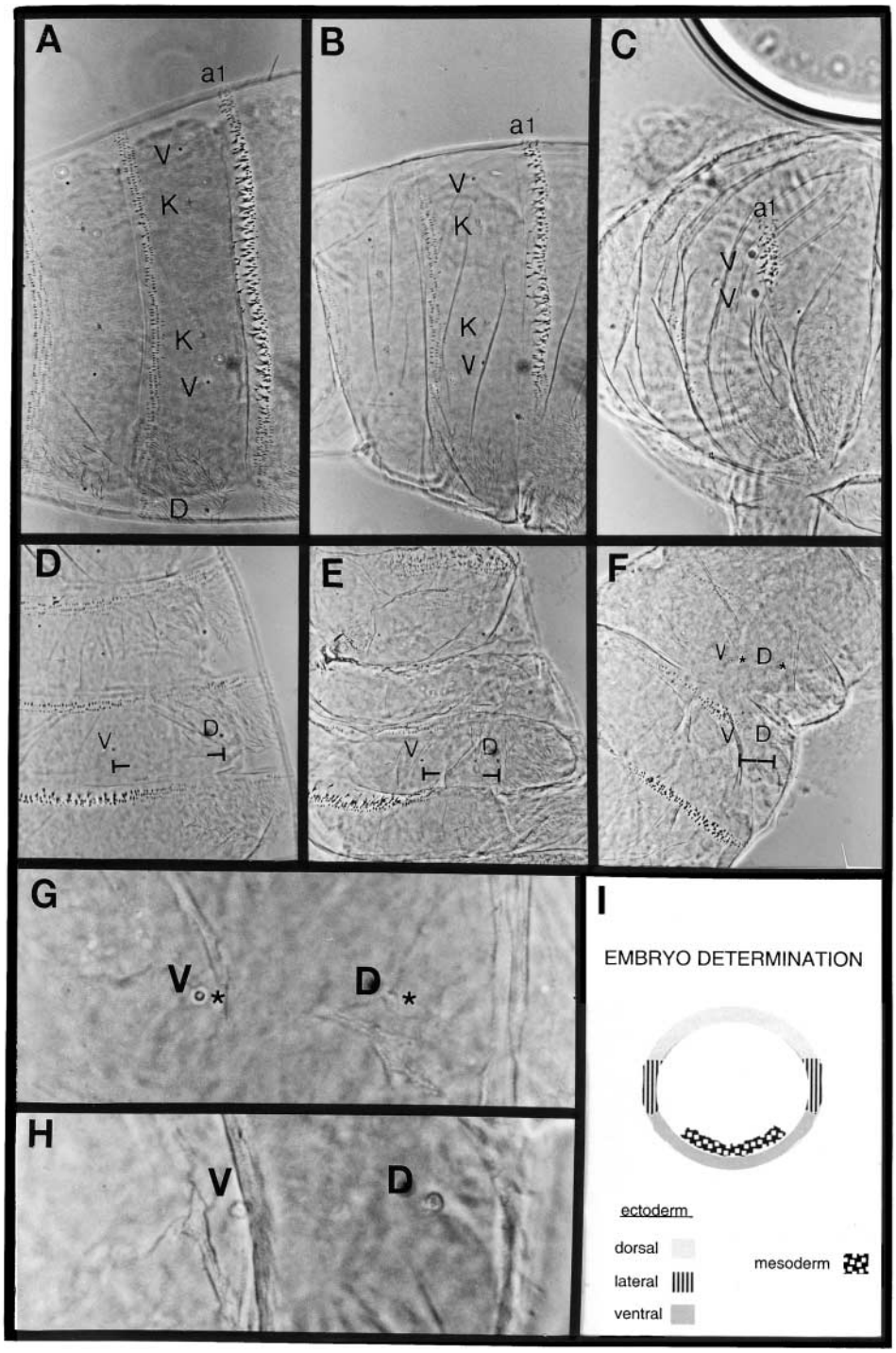

FIGURE 4.—Cuticular preparations of *D-raf* embryos with $D\text{-}raf^{WT}$, $D\text{-}raf^{\Delta 315}$, or $D\text{-}raf^{S743A}$ expression. Views from the ventral or lateral surface of the third thoracic and first abdominal denticle regions are shown. (A) Expression of the $D\text{-}raf^{WT}$ transgene resulted in full rescue of *D-raf* null embryos including Keilin's organs and ventral and dorsal black dots. Expression of the $D\text{-}raf^{S743A}$ or $D\text{-}raf^{\Delta 315}$ gene resulted in partial rescue of the *D-raf* null phenotype. Both (B) $D\text{-}raf^{S743A}$ and (C) $D\text{-}raf^{\Delta 315}$ embryos showed pattern deletions in the ventral epidermis, which was most severe for embryos expressing the D-raf $^{\Delta 315}$ protein. In this case, deletion of Keilin's organs was accompanied by an expansion of the remaining cellular fates and gave rise to enlarged ventral black dots. For this $D\text{-}raf^{\Delta 315}$ embryo the naked cuticular region between the enlarged ventral black dots and the first abdominal denticle belt appeared narrow. This was not typical of most $D\text{-}raf^{\Delta 315}$ embryos and was likely an artifact of embryo twisting. Lateral views of *D-raf* null embryos with *D-raf* transgene expression: (D) A wild-type organization of ventral and dorsal black dot material was observed after expression of the $D\text{-}raf^{WT}$ transgene. For embryos that developed with (E) D-raf ^{S743A} or (F) D-raf $^{\Delta 315}$ proteins, the distance between ventral and dorsal black dots was reduced. Deletion of lateral cuticle was most extreme for embryos with D-raf $^{\Delta 315}$ proteins. High-magnification views of the lateral surface from the $D\text{-}raf^{\Delta 315}$ embryo shown in F with ventral and dorsal black dots from the second (G) and third (H) thoracic segments. (I) We hypothesize that *D-raf* acts to specify cellular fates in two distinct ectodermal domains of the embryo. Together with the EGF receptor, the D-raf protein acts to determine cell fates in the ventral ectoderm. Laterally, *D-raf* acts with an unknown receptor to elicit lateral ectodermal identities. *a1*, abdominal denticle belt one; *D*, dorsal black dot; *K*, Keilin's organ; *V*, ventral black dot.

and FERGUSON 1999). The Dpp ligand directs nuclei to initiate programs for the development of extraembryonic aminoserosa and dorsal epidermis. In the ventral domain, the EGF receptor acts to determine cells of the neuroectodermal region that give rise to the ventral nerve cord and the final cuticular pattern. The specification of lateral fates that comprise the remaining 20% of the ectoderm occurs in cells that lie between dorsal

and ventral domains and is initiated by an unknown mechanism. These cells secrete cuticular hairs, denticles, or naked cuticle similar to those exoskeletal structures produced by dorsal and ventral cells.

A variety of genetic approaches were employed here to define the role that *D-raf* plays in the development of the embryo. Using a constitutively active D-raf protein we found that *D-raf* acts downstream of the *Egfr* for the specification of ventral ectodermal cell fates. We have also discovered that *D-raf* plays a second role in a novel pathway that is required for lateral cell development. In particular the *D-raf*^{S743A} and *D-raf*$^{\Delta 315}$ alleles generated *in vitro* proved useful in defining the function of *D-raf* in cells of the lateral epidermis. We hypothesize that this novel pathway acts to specify cells of the lateral ectoderm subsequent to instructions received by nuclei from the dorsal maternal gene product. Thus, dorsoventral patterning in the embryo is likely dependent on the activity of three zygotic signaling pathways with *Dpp* that acts in dorsal cells, *Egfr* that directs cells in the ventral ectoderm, and a novel RTK pathway that specifies lateral cell fates.

Determination of lateral cellular fates: The lateral epidermis consists of two narrow strips of tissue on the left and right sides of the embryo extending from the anterior head to the posterior tail region. For the meta- and meso-thoracic regions this lateral tissue gives rise to epidermal cuticular structures that form between dorsal and ventral black dot sensilla (according to Campos-Ortega and Hartenstein 1985). Along the circumference of each abdominal segment these two regions of lateral cuticle can be subdivided into dorsolateral and ventrolateral domains. Normally in late-stage embryos the dorsolateral region is characterized by numerous discontinuous rows of long slender hairs that have a pattern similar to that found for region b of the dorsal epidermis (Campos-Ortega and Hartenstein 1985). These dorsolateral hairs are most similar in size and morphology to a subset of dorsal hairs, the 4° hairs (Heemskerk and DiNardo 1994). The ventrolateral domain is characterized by a segmental organization of naked cuticle alternating with two to three sparse rows of denticles similar to those found in the ventral belts although not as strongly pigmented.

Several findings have indicated that a novel pathway acts in the determination of lateral ectodermal cell fates and were consistent with a role for *D-raf* in this pathway. Irish and Gelbart (1987) found that embryos that developed in the absence of *dpp* and *dorsal* activity were lateralized. Mutations in the Drosophila *dCREB-A* gene are also important for defining lateral embryonic regions. In the absence of *dCREB-A* gene function, embryos showed development of only lateral epidermal structures (Andrew *et al.* 1997). *dCREB-A* encodes a transcription factor that is required in both *Dpp* and *Egfr* signaling cascades. *dpp* is a member of the TGFβ family, while *spitz* is a TGFα family member and potentiates *Egfr* signaling in the ventral ectoderm (for review see Schweitzer and Shilo 1997; Podos and Ferguson 1999). Two consequences of lateral cell induction were also identified: activation of the MAP kinase protein and expression of the *msh* gene encoding a homeodomain protein product (Skeath 1998; Yagi *et al.* 1998; von Ohlen and Doe 2000). Using D-raf proteins with partial function we have found that *D-raf* also participates in the development of the lateral epidermis most likely to specify cellular fates in the lateral ectoderm.

Is there a RTK receptor responsible for triggering the activation of the D-raf protein and MAP kinase in cells of the lateral ectoderm? One possible candidate is the insulin receptor (*Inr*) gene product. It was noted that occasionally embryos developing without zygotic *Inr* activity showed a decrease in denticle belt width (Fernandez *et al.* 1995). To better address the potential role of *Inr* signaling in lateral ectodermal patterning, a phenotypic analysis of *Inr* mutant embryos derived from eggs lacking maternal contributions of *Inr* would likely be informative. Alternatively, the generation of protein null alleles of *Inr* may help to better define the function of this RTK receptor in the embryo. In mammalian systems, mitogenic signaling by insulin in fetal rat, brown adipocyte, and primary cultures involves the activation of Ras and Raf-1 proteins (Valverde *et al.* 1996). Insulin was also known to trigger an increase in Raf-1 activity in several cell lines that expressed large numbers of insulin receptors (Blackshear *et al.* 1990; Kovacina *et al.* 1990).

A gradient of *D-raf* activity specifies ventral ectodermal cell fates: The Raf-MEK-MAP kinase cascade acts in a variety of cells to transmit RTK-generated signals during Drosophila development. Here we show that the protein kinase activity of *D-raf* was required to elicit distinct ventral cell fates specified by the EGR receptor in early embryos. Using partial loss-of-function mutations in *D-raf*, cell fates normally specified by high levels of *Egfr* activity were lost while those that required lower receptor activity appeared normal or were expanded. Similarly, in *Tor* RTK signaling different levels of *D-Ras*, *D-raf*, or *corkscrew* activity were shown to have distinct transcriptional and morphological consequences in embryonic head and tail development (Baek *et al.* 1996; Greenwood and Struhl 1997; Radke *et al.* 1997; Ghiglione *et al.* 1999).

How is a graded pattern of cell types within a developmental field generated by an RTK receptor? It has been hypothesized that the main function of the Raf-MEK-MAPK phosphorylation cascade is to amplify RTK-initiated signals. In this case, the quantity of activated Raf, MEK, and MAPK molecules is directly proportional to the number of receptor molecules activated, in the absence of feedback mechanisms. This information is then translated into position-dependent gene expression patterns that lead to morphological changes and cellular

development. In this model, the quantity of activated RTK receptors defines the determined state of the cell. However, a number of studies in Drosophila reveal the existence of parallel signaling pathways emanating from a receptor during embryonic development (Hou *et al.* 1995; Raabe *et al.* 1995; Herbst *et al.* 1996). To extend the amplification hypothesis, the Raf-MEK-MAP kinase cascade may also act to integrate signals received from these parallel pathways and ultimately define precise transcriptional outcomes using a multistep mechanism. In mammalian cells, Raf-1 is regulated by a variety of inputs including the enzymatic function of PKC, Src, and Jnk kinases that upregulate activity (for review see Morrison 1994, 1995; Morrison and Cutler 1997). Autophosphorylation also plays a role in regulating Raf-1, as well as binding to Ras, 14-3-3, KSR, hsp90, and p50 proteins. In addition, PKA, Atk (PKB), and phosphatases have been implicated in the downregulation of Raf-1 function (Cook and McCormick 1993; Wu *et al.* 1993; Hafner *et al.* 1994; Rommel *et al.* 1999; Zimmermann and Moelling 1999).

Here we address the consequences of eliminating key *D-raf* regulatory domains or specific serine residues that might act to integrate distinct signaling pathways in the *Egfr* pathway for ventral cell determination. In general, signal transmission was less reliable for D-raf proteins that lacked the negative regulatory site S388 (D-raf^{S388A}) or the regulatory sequences CR1 and CR2 associated with the N-terminal one-half of the molecule (D-raf$^{\Delta 445}$). However, both proteins showed the potential to transmit the highest level of ventral signal. This phenomenon was perhaps indicative of an important role played by the D-raf protein in the assembly of multiprotein complexes with components derived from parallel pathways. The full-length wild-type D-raf molecule, which contains several conserved motifs, may serve to bring parallel-signaling components together. Thus, the structural integrity of the D-raf protein may be important for the efficiency of complex assembly or its stability. In this model only complete and stable-signaling complexes achieve the highest level of signal output. We speculate that in the case of D-raf^{S388A} and more often for D-raf$^{\Delta 445}$ proteins complete signaling complexes were not built, leading to the phosphorylation of fewer D-MEK molecules, decreased signal output, and fewer cell fate choices specified within the *Egfr* developmental field.

In contrast, the *Egfr* signal was severely compromised when transmitted by either D-raf^{S743A} or D-raf$^{\Delta 315}$ proteins. The range of cell types specified by these mutant D-raf molecules was dramatically reduced from the wild type. In both cases, the establishment of cell fates that require the highest level of *Egfr* activity was consistently lost. Serine 743 may be important for the formation of D-raf dimers or oligomers as has been suggested for Raf-1 (Farrar *et al.* 1996; Luo *et al.* 1996; Morrison and Cutler 1997). This type of complex may be essential for the generation of the highest level of ventral signal. In embryos that developed with D-raf$^{\Delta 315}$ proteins, cell fates were generated that required substantially lower levels of *Egfr* activity. We speculate that the wild-type D-raf protein undergoes release from negative regulation imparted by the CR2 domain via its N-terminal and CR1 sequences. In the case of the D-raf$^{\Delta 315}$ protein, maintenance of the negative regulatory function of CR2 severely limited the ability of D-raf molecules to activate D-MEK. These results point to a multistep process in the generation of active D-raf molecules with multiple upstream factors acting in parallel. The highest level of D-raf signal was generated when all inputs were received. In the absence of one or several interactions the signaling potential of the D-raf protein was reduced, but not abolished.

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