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Localization of the DER/*flb* protein in embryos: implications on the *faint little ball* lethal phenotype

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Summary

Antibodies were raised against the *Drosophila* EGF receptor homolog (DER) and used for immunohistochemical analyses of *Drosophila* embryos. We found that DER is localized in a wide array of embryonic tissues, displaying a dynamic pattern of expression. DER appears to be expressed in all cells at the cellular blastoderm and gastrula stages. In extended-germ-band embryos, it is found predominantly in the mesoderm and the head. Finally, in retracted-germ-band embryos, DER immunoreactivity is most pronounced at sites of somatic muscle attachments and along the ventral midline of the CNS. We have thus observed that DER is expressed in the diverse tissues which are affected in the DER *faint little ball* (*flb*) embryonic lethal phenotype. The different pattern and extent of expression in each tissue suggests that the disparate aspects of the *flb* phenotype may result from different mechanisms of DER function. To understand the basis for the CNS phenotype of DER/*flb* mutants, we have closely followed the collapse of the CNS in mutant embryos. Our observations on the evolution of the final CNS phenotype, in combination with the temporo-spatial pattern of appearance of DER in the ventral neuroepithelium, suggest that this receptor participates in the second phase of neuron–glia interactions, namely in stabilization of the ladder-like CNS scaffolding formed by outgrowth of pioneer axonal processes along the glial pre-pattern.

Key words: *Drosophila* embryonic development, tyrosine kinase receptors, *faint little ball*, CNS midline, muscle attachments.

Introduction

The *faint little ball* (*flb*) locus was first recognized in a screen for embryonic lethal loci on the second chromosome of *Drosophila* (Nusslein-Volhard *et al.* 1984). Cuticles of unhatched homozygous *flb* embryos display characteristics indicative of several, not obviously related, defects. In cuticles of *flb* embryos, ventral denticle bands are missing or reduced while dorsal hairs are unaffected, suggesting preferential involvement of the ventral epidermis. Absence of the head skeleton points to defects in the cephalic region. Finally, the rounded shape of the cuticle is a manifestation of the failure of the extended germ band to retract. Further characterization of *flb* embryos has indicated that initial normal formation of head structures is followed, beginning at germ band extension, by secondary deterioration in the anterior region (Schejter and Shilo, 1989). The use of antibodies to visualize the central nervous system has also demonstrated that the CNS of *flb* embryos that have failed to retract is collapsed along the midline, displaying fused longitudinal connectives and horizontal commissures (Schejter and Shilo, 1989). Taken together, these results suggest that the *flb* locus encodes an essential protein, which fulfills a wide range of functions during *Drosophila* embryogenesis.

The *flb* locus encodes the *Drosophila* homolog of the vertebrate EGF receptor (Schejter and Shilo, 1989; Price *et al.* 1989), a transmembrane tyrosine kinase receptor that we have previously characterized (Livneh *et al.* 1985; Schejter *et al.* 1986). As a tyrosine kinase, the *Drosophila* EGF receptor, termed DER, is a member of a diverse gene family highly represented in vertebrate species (Hunter and Cooper, 1985; Hunter, 1987; Yarden and Ullrich, 1988) and in *Drosophila melanogaster* (reviewed in Shilo, 1987). DER displays an equal degree of similarity to the vertebrate EGF receptor (Ullrich *et al.* 1984) and *neu* (Schechter *et al.* 1984; Bargmann *et al.* 1986) proteins. The DER protein possesses tyrosine kinase activity (Schejter and Shilo, 1989; Wides *et al.* 1990), demonstrating its functional relatedness to the vertebrate receptors. However, neither the ligand(s) that bind to it, nor the substrate(s) upon which it acts have been identified to date.

The structure of transmembrane tyrosine kinases suggests that they function in transmission of external

signals into the cell. This appears to be the case for three *Drosophila* tyrosine kinase receptors. The *sevenless* protein is crucial for the reception of environmental cues by the eye imaginal disc cells destined to become R7 (Hafen *et al.* 1987). *torso* is required for the generation of terminal structures in the embryo (Sprenger *et al.* 1989). Finally, the DER/*flb* locus was recently demonstrated to be allelic to two additional known mutations, *Ellipse* (Baker and Rubin, 1989) and *torpedo* (Price *et al.* 1989), whose phenotypes suggest that specific developmental information has failed to pass between cells. *Ellipse* (*Elp*), is a dominant 'gain of function' mutation, which gives rise to eyes with a reduced number of ommatidia. The *Elp* phenotype suggests that, in the larval eye imaginal disc, the wild-type receptor is involved in the transmission of an inhibitory signal between cells, which determines the proper number of cells that will initiate differentiation as photoreceptor preclusters. Homozygous *torpedo* (*top*) females are sterile due to the development of ventralized eggs and egg shells. Analysis of the *top* mutation has demonstrated that the gene product is required in the somatic follicle cells surrounding the germ line-derived egg, but not in the egg itself (Schupbach, 1987). DER thus appears to be involved in communication within the egg chamber responsible for determination of dorso-ventral polarity, specifically in the reception of signals from the egg by the follicle cells.

The roles of DER at later stages of development suggest that its earlier embryonic functions may also involve intercellular communication. The different tissues that are affected in the DER/*flb* phenotype suggest that the DER protein will be expressed in a diverse array of embryonic cells. This notion is supported by the *in situ* hybridization pattern to DER RNA during embryonic development, which shows that transcripts are present in most embryonic tissues (Schejter *et al.* 1986; Kammermeyer and Wadsworth, 1987). The pleiotropic nature of the embryonic *flb* phenotype raises several questions. Do the defects in mutants arise from aberrant differentiation of embryonic tissues, or do they reflect cell death and tissue disintegration? Is there a different basis for each aspect of the phenotype? Does DER participate in the same signal transduction pathway in the various embryonic tissues, or do the disparate manifestations of the phenotype stem from subtle differences in the pathway, such as the recognition of divergent ligands and/or substrates by DER?

This paper describes the generation of anti-DER antibodies, and their utilization to monitor the localization of the DER protein during embryonic development. We show that the protein is expressed in a wide array of tissues that coincide with those affected in *flb* mutants. Our results provide support for the notion that DER participates in the transmission of developmental signals crucial to cell determination (in the ventral epidermis) and to cell viability (in the head and CNS). We show that in mutant *flb* embryos the CNS develops normally, and the severe CNS phenotype ultimately observed is the result of secondary collapse.

Materials and methods

Fly lines

Fly strains bearing the Df(2R)PK1, which is deficient in the DER gene, and the JE1 mutant DER allele were provided by J. O'Donnell. A second allele, 2C82, was provided by C. Nusslein-Volhard and E. Wieschaus. Strain 242, with an 'enhancer trap element' (Wilson *et al.* 1989) inserted into the third chromosome, and strain 5704 with the P [ry, *ftz*/lacZ] element on the third chromosome (Hiromi *et al.* 1985) were both the kind gifts of Y. Hiromi and C. S. Goodman. These elements were crossed into the fly strains bearing a mutant DER allele (JE1 or 2C82) to yield strains that were balanced for the mutant DER allele on the second chromosome and homozygous for the transposable element on the third. When not otherwise specified, Canton-S flies were used.

Preparation of antibodies

Polyclonal antibodies were prepared against a trpE/DER fusion protein by inserting a restriction fragment from a DER cDNA clone into the appropriate pATH vector (Dieckmann and Tzagoloff, 1985). To generate a construct encoding amino acid residues 113–404 of the DER extracellular domain, a DER cDNA clone was grown in methylation-deficient bacteria and the *Bcl*I–*Bam*HI fragment, including the first cysteine-rich domain, was inserted into the pATH 1 *Bam*HI site.

Following transformation of *E. coli* DH1 cells with the pATH fusion vector, the fusion protein was induced and partially purified by the following procedures. Typically, 1 ml of overnight starter cultures of transformed bacteria was added to 100 ml M9 medium with 0.5% casamino acids, 10 $\mu g\,ml^{-1}$ thiamine, 0.2% glucose and ampicillin. After 2 h shaking at 33 °C under well-aerated conditions, bacteria were induced by the addition of freshly prepared beta-indoleacrylic acid (Sigma) dissolved in ethanol (5 $\mu g\,ml^{-1}$ final concentration) and left shaking overnight. The following day, bacteria were spun down, resuspended in 2 ml of phosphate-buffered saline (PBS) and lysed by boiling in a 1:1 suspension with sample buffer (50 mM tris, pH 7.0, 25% glycerol, 2% beta-mercaptoethanol, 2% SDS and bromophenol blue). The cleared suspension was loaded onto preparative 10% SDS–polyacrylamide gels. The induced fusion protein was located by lightly staining the gels with Coomassie blue. The portion of the gel containing the protein of interest was excised and minced. The protein was eluted by diffusion into PBS 0.1% SDS for 48 h.

Inoculation of two guinea pigs with the *Bcl–Bam* fusion protein was initiated by injection of about 50 μg of protein (determined by the Bio-Rad Protein Assay) with complete Freund's adjuvant into the foot pad. Two boosts of about the same amount of protein in incomplete Freund's were given at one month intervals. Bleedings began one month after the initial injection.

Antisera were assayed for their ability to immunoprecipitate the DER protein that was produced by *in vitro* transcription and translation in the following manner. A plasmid (SP-DER) containing the full DER type II cDNA minigene construct under the control of the SP6 promoter was prepared by inserting the DER minigene from pDER (Wides *et al.* 1990) into the *Eco*RI site of pSP65 (Melton *et al.* 1984). The DER minigene was constructed by combining a cDNA sequence encoding 80 bp of 5′ untranslated sequence, the type II 5′ alternative exon, and the three common exons following it, with a genomic fragment encoding the large 3′ exon.

RNA transcripts were produced with the Amersham SP6 *in*

vitro transcription system using circular SP-DER template. *In vitro* translation of SP-DER RNA was performed by using 6 μl of micrococcal nuclease-treated rabbit reticulocyte lysate (kindly provided by A. Zilberstein) in a 25 μl mixture containing 200 ng SP-DER RNA, 8 mM phosphocreatine, 20 μg ml^{-1} creatine kinase, 0.2 mM $MgCl_2$, 0.4 mM DTT, 20 mM Hepes, pH 7.6, 70 mM potassium acetate, 0.3 mM spermidine, and 20 μCi [^{35}S]methionine (1115 Ci $mmol^{-1}$, Amersham). Reactions were run for 60 min at 30°C followed by a 1 mM cold methionine chase for 5 min. 1 μl of antiserum was incubated with 2.5 μl of *in vitro* translation product in 200 μl RIPA and immunoprecipitated as described below.

Before use in immunohistochemistry, the guinea pig antiserum that gave the highest initial staining signal was affinity-purified by passage over an Affigel 10 plus Affigel 15 column (Bio-Rad) in a ratio of 3:1, linked to bacterial proteins and an unrelated trpE fusion protein. The eluate was collected and further purified by absorption against 0–2 h fixed embryos.

Immunohistochemistry of whole mount embryos

For immunocytochemistry involving fluorescent-conjugated secondary antibodies, dechorionated embryos were fixed for 20 min in 3.7% paraformaldehyde in PEM (0.1 M Pipes, 2 mM EGTA, 1 mM $MgSO_4$, pH 6.95) saturated with an equal volume of heptane. Vitelline membranes were removed by replacing the aqueous layer with methanol and shaking vigorously. Embryos that 'cracked' out of their vitelline membranes and sank to the bottom of the tube were washed 3 times with methanol and 3 times with PBT (1×PBS, 1% BSA, 0.1% Triton X-100). They were then blocked for 4 h at room temperature with PBT containing 10% BSA. After an additional wash with PBT, embryos were incubated overnight at 4°C with a 1:200 dilution (in PBT) of guinea pig anti-DER amino terminal antiserum, preabsorbed as described above.

The following day the embryos were washed 5 times quickly in PBT. They were then incubated for 2 h at room temperature with a 1:300 dilution of rhodamine-conjugated affinity-purified goat anti-guinea pig antibody (Jackson Immunoresearch Laboratories), which had been pre-absorbed against fixed embryos. In the case of double labeling with anti-HRP, fluorescein-conjugated goat anti-HRP (Cappel) was included at this time at a 1:300 dilution in PBT. The embryos were then washed as above and mounted in 90% glycerol, 10% PBS. They were viewed with a Nikon Microphot-FX microscope under fluorescence and phase optics. Pictures were taken with T-Max 400 film, with timed 0.5 min exposures for immunofluorescent pictures.

For immunocytochemistry involving peroxidase-conjugated secondary antibodies, dechorionated, devitellinized and washed embryos were blocked for 4 h in normal goat serum (50 μl NGS ml^{-1} in PBT). They were then washed and treated with antibody diluted in PBT plus NGS. Depending on the experiment, the primary antibody was guinea pig anti-DER (at 1:200 dilution), mouse monoclonal anti-BP104 (at 1:2 dilution, from A. Bieber, N. Patel and C. S. Goodman) or mouse monoclonal anti-beta-galactosidase (at 1:1000 dilution, Promega). The following day, after 5 washes, the embryos were blocked again for 0.5 h in NGS, as above, and then incubated for 2 h in peroxidase-conjugated affinity-purified secondary antibody (Jackson Immunoresearch Laboratories), preabsorbed against fixed embryos and diluted 1:300 in PBT plus NGS. After washes as above, embryos were incubated for 10 min in 200 μl of PBT plus 100 μl of DAB (1 mg ml^{-1} DAB, Sigma, in PBT). 3 μl of a 3% solution of H_2O_2 was added and the reaction was allowed to proceed for 15 min. The embryos were then washed twice with PBT, dehydrated in ethanol and allowed to clear overnight in methyl salicylate. They were mounted the following day and viewed by Nomarski optics. Slides were taken with Ektachrome tungsten160 film.

Results

Immunolocalization of the DER protein in embryos

The embryonic DER/*flb* phenotype suggests that the protein plays diverse roles in embryogenesis. It was therefore of interest to follow the localization of DER during embryonic development. The embryonic expression of the DER gene product was studied by staining embryos with guinea pig antibodies raised against the extracellular domain of DER and preabsorbed against both nonspecific bacterial proteins and 0–2 h *Drosophila* embryos. We are confident that the immunoreactivity of the purified guinea pig anti-DER antibody is solely due to the DER gene product, because PK1 embryos, homozygous for a deficiency of the DER locus and identified as such by their *faint little ball* phenotype (Fig. 1), were devoid of any specific signal.

The guinea pig anti-DER antiserum did not stain unfertilized eggs or wild-type embryos that had not yet undergone cellularization (not shown). The DER gene product is first observed by immunostaining after cell formation. It is widely distributed throughout the cellular blastoderm in a manner that suggests that it is localized at the periphery of cells in the newly formed plasma membranes (not shown). The initial emergence of the epitopes recognized by this antiserum thus coincides with the first appearance of DER transcripts previously visualized by *in situ* hybridization (Schejter *et al.* 1986), and agrees with Northern analysis indicating that there is no maternal component of DER transcripts (Lev *et al.* 1985).

During gastrulation, DER continues to be expressed in all ectodermal epithelial cells (Fig. 2B). When embryos are observed at the focal plane of the yolk, rather than at the surface, the density of DER appears to be highest in the basal portions of these cells, including the basal membranes (Fig. 2C). While the pattern of DER ectodermal expression remains largely uniform at this stage, hints of banding can be discerned that are perhaps associated with the deepening cephalic furrow and anterior and posterior transverse folds (Fig. 2B). Especially intense immunofluorescence is observed in association with epithelia that take on a high columnar configuration such as the epithelium invaginating to form the posterior discoid plate, which carries the pole cells towards the dorsal side of the embryo and gives rise to the posterior midgut invagination (Fig. 2B). The pole cells themselves, however, do not appear to express the DER protein. At this stage, hazy immunofluorescence is also visible in the ventral part of the embryo above the ventral furrow (Fig. 2B).

In the germ-band-extended embryo, continued DER expression in the ectoderm is overshadowed by the bright immunofluorescence visualized in an internal layer of cells, which exhibits a clear pattern of metamery (Fig. 3A). In order to identify the cell layer that

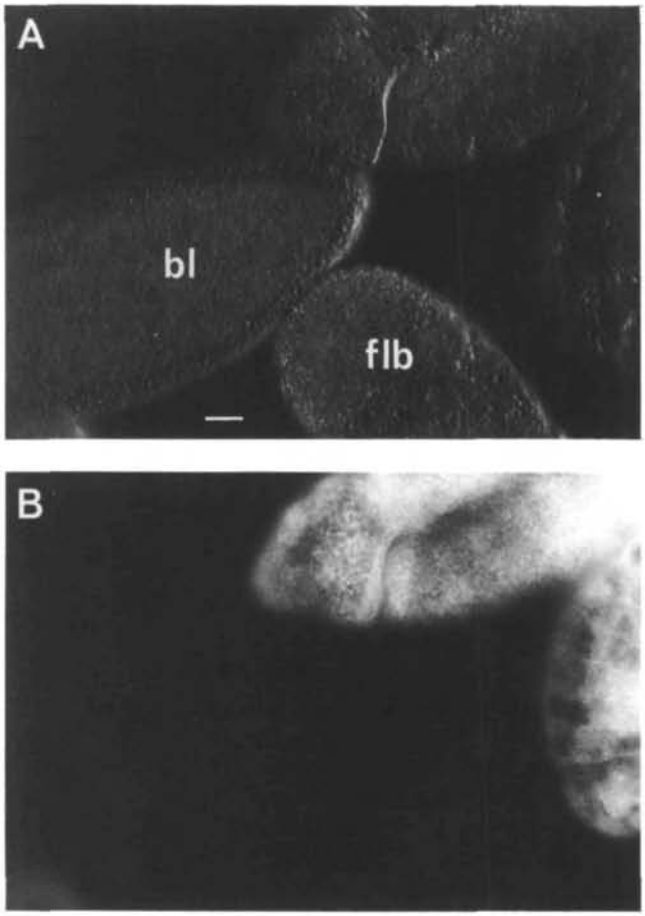


Fig. 1. Immunohistochemical staining showing the specificity of the anti-DER antibody. (A) Phase contrast. (B) Immunofluorescent image of A. The figure shows a mixed population of wild-type and *flb* embryos, stained with a primary guinea pig anti-DER antibody, and a secondary rhodamine-conjugated goat anti-guinea pig antibody. (Unless otherwise stated, all embryos shown in Figs 2–4 were also stained in the same way.) The two wild-type embryos in the upper right corner are brightly immunofluorescent. The lower right embryo displays the *faint little ball* (*flb*) phenotype and does not stain. On the lower left, a cellular blastoderm stage embryo (bl) in which the phenotype due to DER deficiency is not yet apparent, is also not immunoreactive. Bar=50 μm. bl, blastoderm; flb, *faint little ball*.

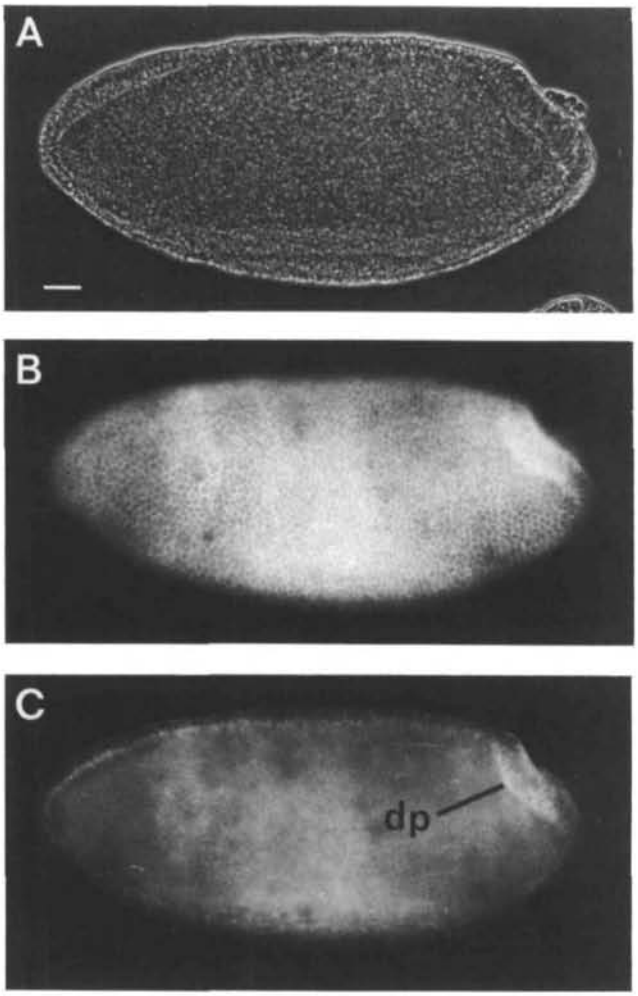


Fig. 2. DER expression in gastrulating embryos. In this figure and Fig. 3, anterior is to the left and posterior to the right. Dorsal is up and ventral down. (A) Phase contrast, note the pole cells at the posterior surface of the embryo. (B) Surface view. Individual cell outlines can be seen. (C) View at the level of the yolk, showing bright immunofluorescence in the high epithelium of the dorsal plate (dp) which carries the pole cells dorsally. Bar=50 μm. dp, dorsal plate.

expresses DER, embryos were double stained with anti-DER antibodies followed by a rhodamine-conjugated secondary antibody (Fig. 3B) and fluorescein-conjugated anti-HRP antibodies (Fig. 3C). By comparison of Fig. 3B with Fig. 3C, it can be seen that the cell layer that is immunoreactive with the anti-DER antibody is internal to the neuronal layer recognized by the anti-HRP antibody (Jan and Jan, 1982). Therefore DER appears to be localized in the newly formed mesodermal cell layer which has invaginated during gastrulation and is becoming segmented at this stage.

In addition to staining of the trunk ectoderm and mesoderm, we always observed very intense immunofluorescence in the head region (Fig. 3A). This can be seen in the mandibular bud (immediately anterior to the cephalic furrow), the procephalic lobe, and, most prominently, in the clypeolabrum. The clypeolabrum is distinguished by its high cylindrical epidermis, unlike the flat cells of the rest of the procephalon, and is also the site where cephalic mesoderm is found. Since staining of the clypeolabrum is both peripheral and internal, it is likely that the immunoreactivity we observe in the head of the germ-band-elongated embryo is at least partly associated with the cephalic mesoderm.

The final embryonic pattern of DER expression becomes defined during germ band shortening and subsequent stages (Figs 4 and 5). The epidermis at the tip of the clypeolabrum is still intensely immunofluorescent (Fig. 4A). Also intensely immunofluorescent, but to a lesser degree, is the epithelium of the terminal

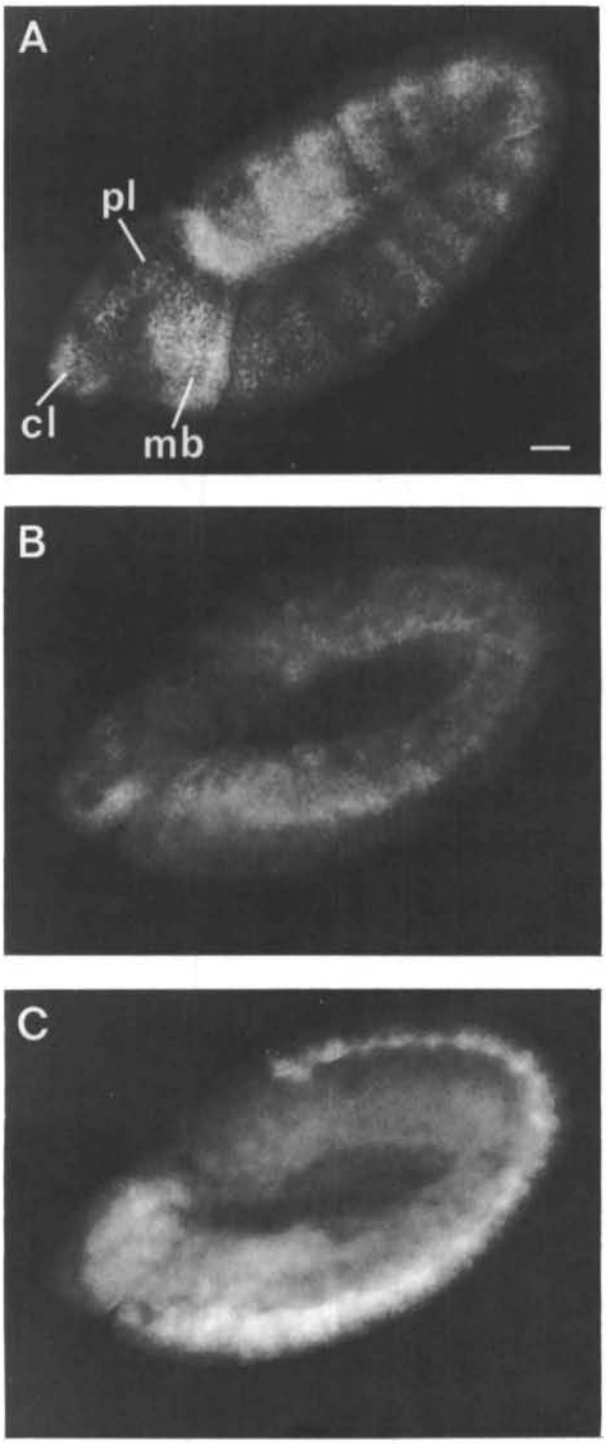


Fig. 3. DER expression in germ-band-elongated embryos. (A) Surface view showing metameric staining in the trunk and widespread expression in the head, in the mandibular bud (mb), procephalic lobe (pl) and clypeolabrum (cl). (B) Internal view of a different embryo. (C) Same embryo as in B, double stained with fluorescein-conjugated anti-HRP antibody. Note that the cell layer in the trunk that is recognized by the anti-DER antibody is internal to the cells expressing neural antigens. Bar=50 μm. cl, clypeolabrum; mb, mandibular bud; pl, procephalic lobe.

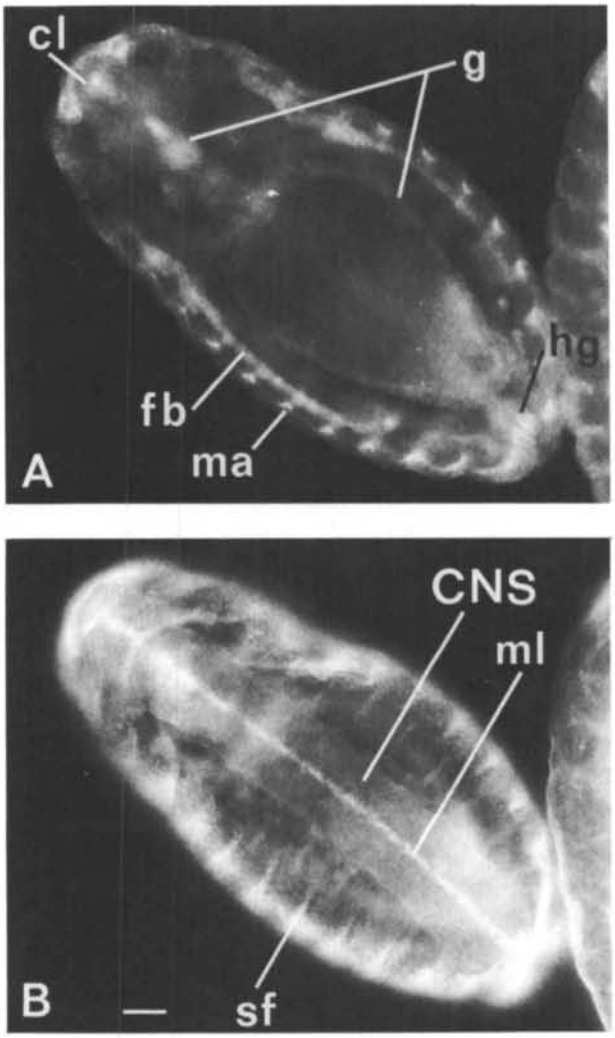


Fig. 4. DER expression in germ-band-shortened embryos. Anterior is to the left and posterior to the right. (A) Internal view, showing immunoreactivity in the clypeolabrum (cl) and hindgut (hg), along the outline of the gut (g), in the fat body (fb) and in somatic muscle attachments (ma). (B) Ventral view, showing staining of the segmental furrows (sf) causing a criss-crossing pattern across the embryo, and bright immunofluorescence along the midline (ml) of the CNS. Bar=50 μm. CNS, central nervous system; cl, clypeolabrum; fb, fat body; g, gut; hg, hindgut; ma, muscle attachments; ml, midline; sf, segmental furrow.

portion of the hindgut (Fig. 4A), which is also of ectodermal origin. DER epidermal immunoreactivity in the thorax and abdomen becomes localized primarily to the segmental grooves. The epidermal staining tapers off on either side of the grooves. This periodic nature of staining with the anti-DER antibody results in what can be seen at the surface focal plane as arch-like immunofluorescence criss-crossing the face of the embryo (Fig. 4B).

Intimately related to the segmental furrows are the muscle apodemes (Figs 4A and 5A). These are the focal attachment sites in thoracic and abdominal segments of the intersegmental muscles. The formation of these

somatic muscles occurs during stages 13–15 (Campos-Ortega and Hartenstein, 1985), by fusion of individual somatic mesodermal cells to form syncytia. Insertion of muscles at apodemes and subsequent stretching occurs at stages 16–17. DER expression at these muscle apodemes is striking. Localization of DER seems to be primarily in tendon cells at the ectodermal epithelial aspect of the apodemes. The focal planes of Figs 4A and 5A were chosen to emphasize the concentration of DER at these points of attachment.

Most splanchnic mesodermal derivatives appear to continue to express DER at this stage. Thus DER can be discerned in the fat body extending from the head to the gonads between the gut and somatic musculature (Fig. 4A). In addition, DER immunoreactivity is readily apparent outlining the contours of the alimentary canal from the pharynx in the head (including the pharyngeal muscles), through the terminal segment of the hindgut (Fig. 4A). This pattern of staining is likely to reflect the expression of DER by the visceral musculature which ensheathes the internal body structures. DER expression, however, appears to have been lost from at least some somatic mesoderm derivatives, for example from the intersegmental muscle cells which insert into the muscle attachments in the body wall, as clearly demonstrated in Fig. 5A.

A final feature of DER expression which becomes dramatically manifested in the germ-band-retracted embryos is its presence along the midline of the ventral median cord (Figs 4B and 5B). DER immunoreactivity is discontinuous along the ventral aspect of the embryo in the plane of the central nervous system (Fig. 5B). Staining of the midline with anti-DER antibodies is not associated with the cell bodies of neurons or related supportive cells which lie along the CNS midline (Fig. 5B). The discontinuous pattern of expression along with the absence of a clear periodic pattern suggest that DER immunoreactivity may be associated with the cell processes of a subset of midline glial cells. Comparison of the staining pattern of anti-DER antibody with that of anti-HRP in older *Drosophila* embryos confirms that the nervous system staining with the anti-DER antibody is strictly limited to midline staining, and this antibody does not recognize any other structures in the central or peripheral nervous system (not shown).

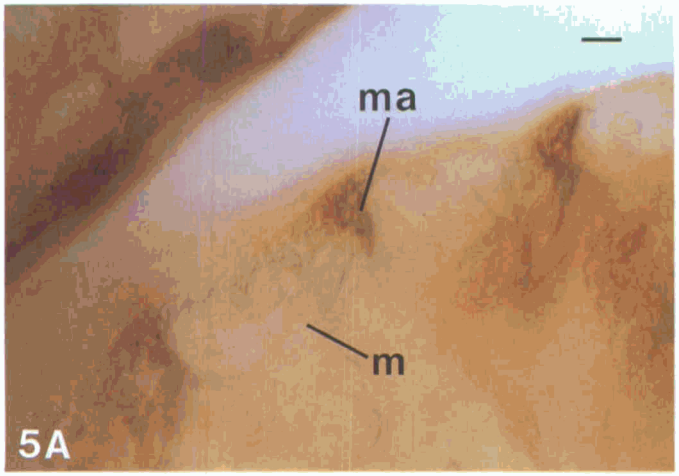
*Further characterization of the collapsed CNS phenotype of DER/*flb *embryos*

The restricted expression of DER in a subset of midline cells in the ventral nerve cord is in striking contrast to the wide range of CNS deformations observed in DER/*flb* mutant embryos. Using FITC-conjugated anti-HRP antibodies to visualize neuronal surfaces, we have previously shown that the segmentally reiterated ladder-like scaffolding of the central nervous system in late DER/*flb* mutant embryos is completely disorganized (Schejter and Shilo, 1989). The commissures that span each neuromere are absent or fused, and longitudinal axon tracts are often fused. Misrouting of segmental and intersegmental nerves is also observed.

Fig. 5. DER expression in muscle attachments and the CNS. Higher magnification of DER expression in embryos slightly older than in Fig. 4, stained with the primary guinea pig anti-DER antibody, and a secondary HRP-conjugated goat anti-guinea pig antibody. (A) DER is present in sites of muscle attachment (ma) to the body wall, probably in tendon cells, but not in the somatic muscles (m). (B) DER is present along the midline (ml) of the CNS. No other immunoreactivity is observed in the CNS or PNS. Bar=10 μm. CNS, central nervous system; m, muscles; ma, muscle attachments; ml, midline; PNS, peripheral nervous system.

Fig. 6. Evolution of the collapsed CNS phenotype and analysis of the midline neuroepithelium in DER/*flb* embryos. (A–D) Embryos stained with antibody BP104 against the nervous system, followed by an HRP-conjugated secondary antibody. A and C are wild type embryos. B and D are DER/*flb* embryos, recognizeable by the deteriorating cells in the cephalic region (arrowheads). A and B are germ-band-extended embryos. C and D are germ-band-retracted embryos. (The peripheral nervous system is also visualized in D because the embryo is slightly older than in C.) (E) Wild type (on the right) and DER/*flb* (on the left) embryos bearing an 'enhancer trap element' which allows visualization of the midline neuroepithelium with anti-beta-galactosidase. (F) A DER/*flb* germ-band-extended embryo bearing a P element directing beta-galactosidase expression under the *ftz* promoter. beta-galactosidase was visualized with anti-beta-galactosidase antibody and can be seen in the seven stripes typical of *ftz* expression and in the paired MP2 cells (arrowhead) which also express *ftz*. (In E and F the DER/*flb* mutant embryos were identified by anteriorly detaching cells and abnormally pronounced parasegmental furrows observed at a different focal plane.) Bar is the same for A–D and F, and different for E. Bar=50 μm.

In light of the restricted expression of DER in the ventral midline of wild-type germ-band-retracted embryos, we chose to closely follow the evolution of the mutant phenotype during formation of the CNS in embryos homozygous for a severe allele of DER (the JE1 allele). To do this, we used monoclonal antibody BP104 (which recognizes a cytoplasmic determinant in the early central nervous system and in all cells of the peripheral nervous system) to stain homozygous JE1 flies. Heterozygous, balanced, JE1 flies were self-crossed and the homozygous offspring were identified at the germ-band-extended stage by the detachment of cells in the cephalic region and the appearance of abnormally pronounced parasegmental furrows. When the developing CNS of germ-band-extended JE1 homozygous embryos is compared with that of similarly staged wild-type embryos (Fig. 6A, 6B), no significant differences can be observed. In the DER mutant embryos, as in the wild type, the horizontal commissures span the ventral midline and join the longitudinal axon tracts. However, when older, germ-band-retracted age embryos are stained, fusion of the longitudinal connectives and loss of the horizontal commissures are apparent in the JE1 homozygotes (Fig. 6D). These observations demonstrate that the severe CNS phenotype in DER/*flb* embryos does not arise as the CNS scaffolding is being generated. Rather, in mutant em-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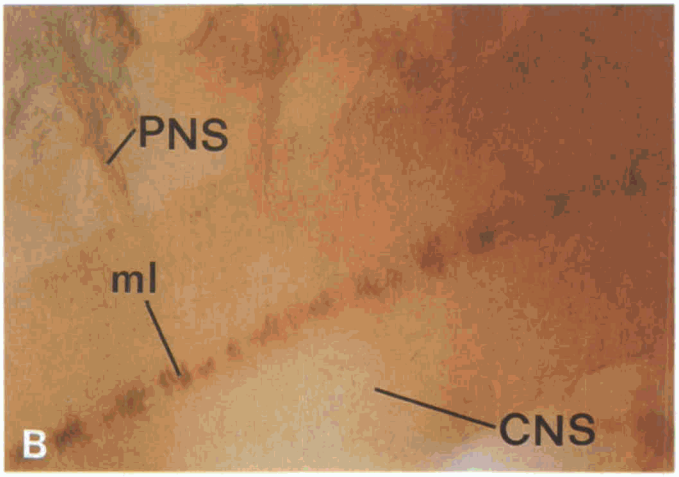
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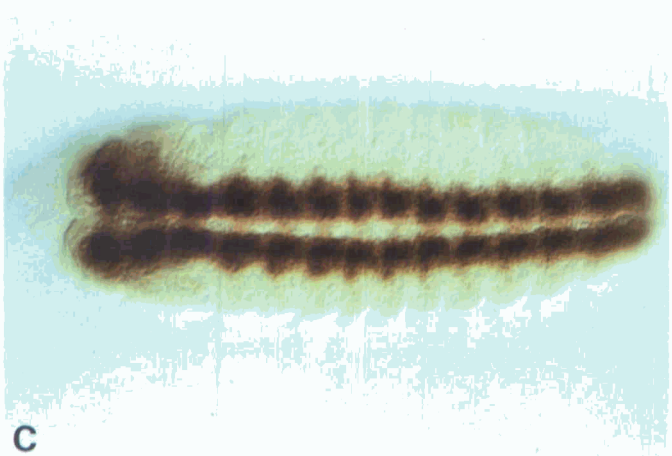
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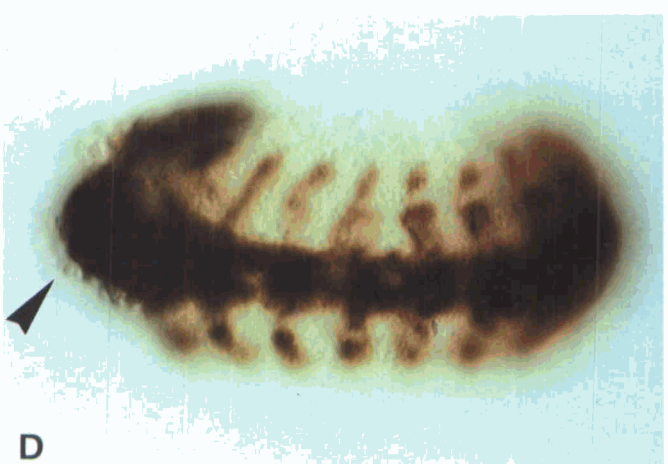
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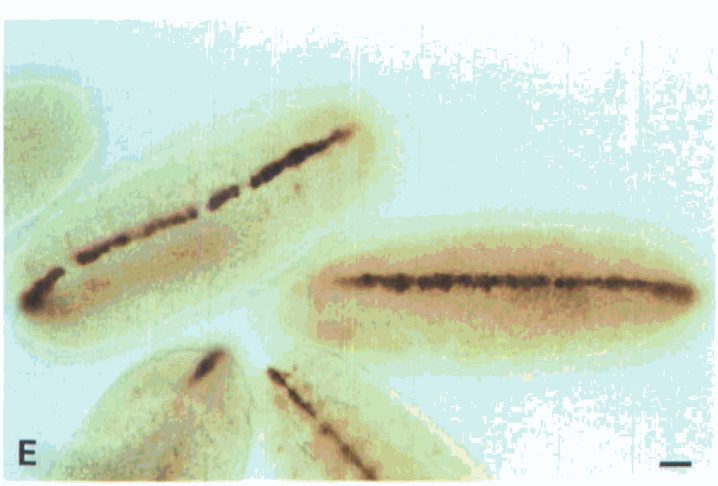
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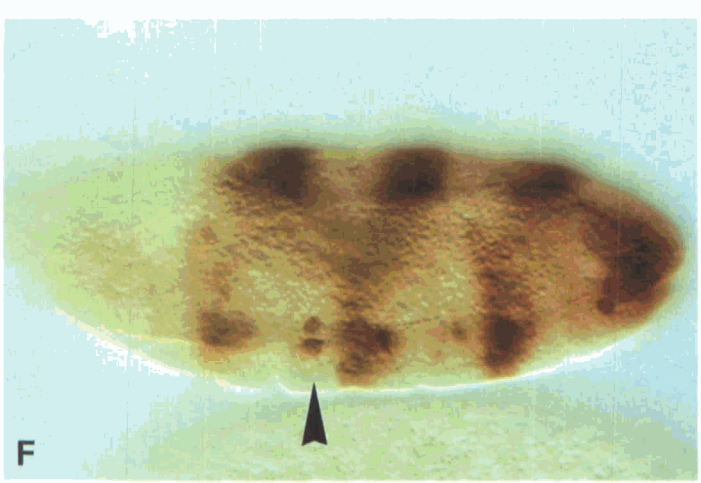
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bryos, the CNS appears to form normally and then to collapse at some later stage.

In order to examine DER mutants for the presence and disposition of various mesectodermally derived midline cell types, two approaches were taken. In one approach, we used an 'enhancer trap' element which has inserted into the third chromosome in a position where endogenous enhancer elements direct the midline-specific transcription of the lacZ gene under a minimal promoter. In self-crosses of flies balanced for the JE1 DER allele on the second chromosome and homozygous for the introduced enhancer trap element on the third chromosome, one fourth of the progeny are homozygous DER mutants (in addition to bearing the enhancer trap element). When the pattern of beta-galactosidase expression is followed by antibody staining of JE1 homozygous embryos, it is found to differ only slightly from the pattern in normal embryos (JE1 heterozygous siblings). While in normal germ-band-extended embryos beta-galactosidase staining is detected in a continuous band along the ventral midline encompassing a restricted set of identified and unidentified neuronal and non-neuronal cell types (Fig. 6E, right hand embryo), staining of DER/*flb* mutants reveals a similar pattern with only a number of discontinuities in the midline neuroepithelial band (Fig. 6E, left-hand embryo). These results indicate that the abnormalities are minimal in the midline cell population of mutant DER germ-band-extended embryos.

In the second approach that was taken, the fate of one specific pair of neurons situated on either side of the midline, the MP2 cells, was followed. Since *ftz* is expressed in these cells (Doe *et al.* 1988), their fate could be monitored in a manner similar to that described above by self-crossing flies heterozygous for the 2C82 DER allele and homozygous for a third chromosome element directing beta-galactosidase expression under the *ftz* promoter (Hiromi *et al.* 1985). In DER/*flb* mutant germ-band-extended embryos, each of the two bilaterally paired MP2 cells of a single neuromere are located at some distance from one another, on either side of the midline (Fig. 6F). These results again demonstrate that midline structures are essentially intact in the germ-band-extended DER/*flb* embryo and no extensive collapse which would bring together paired juxta-midline cells has occurred. We therefore suggest that the final CNS phenotype observed in DER homozygous embryos is due to secondary collapse of the CNS superstructure after initial normal generation of horizontal commissures and longitudinal connectives.

Discussion

Localization of DER in embryos

We have been able to follow the distribution of the DER protein throughout *Drosophila* embryogenesis at a high level of resolution using an anti-DER antibody produced in guinea pig against an extracellular segment of DER. In general, our results are similar to previous *in situ* analyses that indicated that DER transcripts are widely distributed in the embryo. Specifically, we have shown that the initial homogeneous distribution of the DER protein at the cellular blastoderm stage gives way, as development proceeds, to subtle regional differences at ventral furrow formation, and a more pronounced localization to several discrete cell layers and tissues in older embryos.

Since the DER protein is a transmembrane protein with homology to known tyrosine kinase receptors, its first appearance after cellularization of the blastoderm, and apparent localization by immunocytochemistry at cell membranes, is not surprising. Expression of DER by all ectodermal cells persists throughout gastrulation and germ band elongation, but eventually resolves into differential expression at the tip of the clypeolabrum (where it appears to be present at high density from germ band elongation to the end of embryogenesis), at the terminal portion of the hindgut, and in the segmental grooves of the epidermis, particularly at the sites of segmental somatic muscle attachments. The dramatic localization of DER to these muscle apodemes puts it into the same category as the *Drosophila* PS1 and PS2 integrins (Bogaert *et al.* 1987; Leptin *et al.* 1989) and the vertebrate fibronectin receptor (Neff *et al.* 1982), which are found in the adhesion plaques that characterize myoepidermal interactions. In its localization to newly invaginated mesoderm, DER is more similar to PS2, but its ultimate localization in the ectoderm at the epidermal aspect of muscle apodemes is highly reminiscent of the pattern of PS1 expression. Since a basal lamina is present at the muscle–epidermal interface of insects, localization of DER at these sites suggests a possible interaction between DER and some extracellular matrix ligand. This notion is also supported by what appears to be the basal location of DER in ectoderm which is more obvious at earlier developmental stages (see Fig. 2C).

In addition to expression in some ectodermal derivatives, DER is found in the mesodermal cell layer of both the trunk and the head of the germ-band-extended embryo. Expression of DER does not persist in all mesodermal derivatives. It does appear to be present in the fat body and in the visceral musculature which forms a mantle around the foregut, midgut and hindgut of germ-band-retracted embryos. DER immunoreactivity around the contours of the gut is absent in *twist* embryos (unpublished results) in which mesodermal invagination fails to occur (Simpson, 1983; Nusslein-Volhard *et al.* 1984), and can thus clearly be attributed to the mesodermal, rather than endodermal, component of the gut wall. On the other hand, DER is clearly absent from the segmental somatic muscles which insert at focal attachments in the body wall.

At germ band retraction, prominent midline DER immunoreactivity is observed in the vicinity of mesectodermally derived glial cells. This is the only nervous system-associated staining that we observe with the anti-DER antibody. There is no staining in the neural cell bodies of the CNS or in the peripheral nervous system. A puzzling aspect of DER expression in the

midline is the absence of any clear periodicity associated with its discontinuous pattern of expression. This feature may be related to the fact that, at high magnification, DER does not appear to be localized in cell bodies and may thus be localized exclusively in processes of a subset of the midline glial cells. Good candidates for the cells whose processes express DER are the six paired midline glial cells (two anterior, two middle and two posterior) which first serve as a blueprint for guiding outgrowth of pioneer axons along the future horizontal commissures and then themselves send out processes that ensheathe the axonal tracts and function in neuronal support (Jacobs and Goodman, 1989; Fredieu and Mahowald, 1989). This tentative observation will have to be resolved by electron immunomicroscopy.

Correlations between the localization of DER and the embryonic flb *phenotype*

The *faint little ball* embryonic phenotype of amorphic mutants in DER is complex and involves many structures. The most prominent defects can be grouped into four categories: cephalic defects, ventral cuticle abnormalities, failure of the germ band to retract and CNS disorganization. For every class of defects, it is important to ask how the DER phenotype relates to the distribution of the DER protein that we have described. In other words, does the localization of the DER protein in embryos help to understand the *flb* mutant phenotype? We must keep in mind, however, that definitive analysis of the anatomical domains in which DER functions during embryogenesis must await identification and localization of the ligands and substrates that interact with this receptor, since DER activity can only take place where the distributions of all three components overlap.

The cephalic defect is the earliest indication of the *flb* phenotype. It is manifested at the germ-band-extended stage in amorphic DER/*flb* homozygous embryos as slight anterior deterioration and retraction. This defect eventually develops into pronounced disintegration characterized by widespread cell detachment. The *flb* phenotype is first evident at a time of intense cephalic expression of DER in the anterior ventral mandibular region, in the clypeolabrum and procephalic lobe. The extent of DER expression appears to be roughly coincident with the scope of the cephalic phenotype. These observations are consistent with the idea that DER is important for maintenance of tissue integrity in most embryonic head structures.

Because of the massive anterior disintegration, head cuticular structures are absent in the cuticle that is formed by mutant DER embryos. In addition, the segmental cuticular ventral setae are absent or much reduced while the dorsal and dorsolateral hairs are normal. The late epidermal defect which results in these cuticular abnormalities does not appear to result from massive loss or death of the ventral epidermis. When antibodies that recognize different subsets of epidermal cells in the extended germ band stage were used to probe JE1 (a severe DER/*flb* allele) homozygous embryos, we noted that each of the specific subpopulations that express *engrailed*, *ftz*, and *wingless* are normally disposed in the *flb* embryo (Schejter and Shilo, 1989, and unpublished). By this criterion, cell viability and commitment to particular cell fates appears to be normal in the epidermis through germ band extension.

In order to understand further the basis for the ventral cuticle defects, it was of interest to follow the integrity of DER-expressing cells in the ventral region of mutant DER embryos. For this purpose we again chose the JE1 allele which was shown to give rise to a severe *flb* phenotype (similar to that of a null allele) in spite of the fact that it expresses a full-length DER protein which can be visualized by an *in vitro* kinase assay (Schejter and Shilo, 1989). Immunohistochemical analysis of JE1 homozygous embryos reveals that DER-expressing cells are present in the epidermis and mesoderm at the germ-band-extended stage and later on (data not shown). The basis for the cuticular aspect of the DER phenotype is thus likely to be different from the head defects in that it does not involve large-scale loss of cells.

At the time when secretion of cuticular structures by the epidermis is initiated at stage 16, DER epidermal expression is limited largely to the segmental furrows. This is in contrast to earlier stages in which it is found throughout the epidermis. Our interpretation of these data is that a functional receptor is necessary for transduction of the signal for determination or maintenance of the determined cell fate in the ventral epidermis of the thorax and abdomen. Although the effects of DER's influence are manifested only at the end of embryogenesis, when the determined epidermal cells execute their differentiated role, DER is likely to be acting at an earlier stage, prior to synthesis of cuticle. This allows us to attempt to define the 'developmental window' during which DER acts in the epidermis. This window is likely to be from late germ band extension (i.e. after cell determination to express the pair rule and segment polarity proteins we monitored) until the time that DER becomes localized to segmental furrows and is unlikely to be affecting the entire ventral epidermis.

Although DER is also expressed by the dorsal epidermis, no phenotype is observed in dorsal cuticular structures, even in embryos homozygous for null alleles. This may indicate that DER has no function in commitment to elaboration of dorsal cuticle in spite of its expression in that region, or it may be indicative of redundancy in the signals for the development of this domain.

The third aspect of the amorphic DER phenotype is the failure of the extended germ band to retract. The mechanism of germ band retraction is unknown. One hypothesis is that it may involve changes in cell shape (Campos-Ortega and Hartenstein, 1985). In light of the striking localization of DER to the newly invaginated mesoderm in the extended-germ-band embryo, it seems possible that mesodermally expressed DER may play some role in this major morphogenetic event. We have not been able to identify any other characteristics of *flb*

embryos that seem likely to stem from expression of DER in mesoderm or some of the mesodermal derivatives. However, it is possible that some late characteristics of the DER phenotype are masked by earlier defects and embryonic death.

By monitoring the development of the CNS in *flb* embryos with the BP104 antibody, we have shown that the initial near-normal formation of the CNS which is seen in younger *flb* embryos is followed by collapse of the ladder-like scaffolding along the ventral midline in embryos that have failed to shorten. As described above, DER is prominently distributed along the midline of the CNS from germ band retraction, thus closely paralleling in its localization and time of its appearance the dramatic buckling of the nervous system along the ventral axis seen in mutant embryos of this age. Both the configuration of the structures expressing DER (i.e. DER is not found in the round pattern typical of expression in cell bodies) and lack of periodicity of these structures suggest that the receptor is present on the cell processes of a subset of midline cells. These may be the three pairs of midline glial cells, which function first in generation of the commissural neuronal superstructure by guiding the outgrowth of pioneer axons and then in support of these axons which they come to ensheathe (Jacobs and Goodman, 1989; Fredieu and Mahowald, 1989). Taken together, these observations raise the interesting possibility that DER is a receptor that is involved only in the second phase of neuron–glia interactions, those interactions between neurons and the surrounding glial processes which are necessary for maintenance of proper neuronal structure and function. These interactions may be mediated by a distinct set of surface molecules from the various cell adhesion and recognition molecules which are thought to act in initial axon outgrowth. The ongoing axon–glia interactions play a role in nutritional support, electrical isolation and other aspects of neuronal physiology.

The extreme degree of disorganization of the nervous system in severe *flb* embryos that are of germ-band-retracted age (when DER appears in the neuroepithelium) has not allowed us to ascertain whether or not DER protein is present along the ventral midline of mutant embryos. However, we do know that at least some midline neuroepithelial cells are largely unaffected in DER mutant germ-band-extended embryos. These include the median neuroblast and its progeny, which are recognized by anti-*engrailed* antibodies in mutant embryos (Schejter and Shilo, 1989), the juxtamidline MP2 precursor cells in which the expression of *ftz* was followed with a *ftz*-lacZ construct (this work), and the entire midline strip of neuroepithelial cells in which we monitored the appearance of beta-galactosidase driven by an enhancer trap element (this work).

Recently, mutations in two other genes, *single-minded* (*sim*) (Crews *et al.* 1988) and *slit* (Rothberg *et al.* 1988), have been shown to give rise to a collapsed nervous system phenotype that is similar to what is observed in *flb* embryos. Both genes encode proteins that are specifically expressed along the midline of the CNS and eventually become restricted predominantly to the three pairs of midline ectodermal cells, the specialized glial cells which we also believe to be good candidates for the cells with DER-positive processes. Interestingly, the appearance of DER in these cells occurs later than that of *slit*, which in turn lags behind the expression of *sim*, suggesting that the three loci function at different times during midline development. While it is tempting to postulate direct or indirect interactions among the three loci (especially in light of the EGF-like motifs that are present in *slit*), there is as yet no evidence to support this notion. Indeed, our observations of the initial normal emergence of the CNS scaffolding in DER mutants and the separation of the bilaterally paired MP2 cells in an *flb* background (in contrast to *sim* embryos in which the two MP2 cells are always situated adjacent to one another) suggest that DER may be acting in a different developmental pathway. We therefore believe that DER functions later than *sim* and *slit* and participates in stabilization, rather than generation, of the CNS superstructure through supportive neuronal–glial interactions.

Comparison of the embryonic localization of DER with different aspects of the severe *flb* phenotype leads us to the conclusion that the pleiotropic nature of the phenotype reflects multiple functions that DER appears to carry out in different tissue types during embryogenesis. Surprisingly, one process in which DER does not appear to take a significant part is proliferation. This has already been suggested on the basis of the DER/*flb* phenotype which becomes apparent only after most cells of ectodermal origin have ceased to divide (Schejter and Shilo, 1989). The immunohistochemical staining described here offers additional indications for the later functions of DER in transmission of signals essential for maintenance of tissue integrity of cells actually expressing the protein (in the cephalic region), in reading of developmental cues that lead to the eventual execution of differentiated cell functions (in the ventral epidermis), and in stabilization of the CNS superstructure, probably by participation in supportive neuron–glia interactions. Whether these diverse roles of DER employ the same signal transduction pathway at the ligand and substrate ends is a crucial question, which should be addressed by further biochemical and genetic analyses.

The disparate roles of DER during embryogenesis discussed here are only a subset of its biological functions. DER transcripts were also shown to be present at later stages of development. In addition, genetic analysis has revealed that the *Ellipse* and *torpedo* loci are also allelic to DER, implicating DER's central role in morphogenesis of the compound eye and the structure of the egg shell and embryo. The availability of antibodies recognizing the DER protein should allow us in the future to follow the distribution of the protein at later stages of development and ask how it pertains to these mutant phenotypes.

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