Development 133, 217-227 doi:10.1242/dev.02198

Drosophila Dok is required for embryonic dorsal closure

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Embryonic dorsal closure (DC) in *Drosophila* is a series of morphogenetic movements involving the bilateral dorsal movement of the epidermis (cell stretching) and dorsal suturing of the leading edge (LE) cells to enclose the viscera. The Syk family tyrosine kinase Shark plays a crucial role in this Jun amino-terminal kinase (JNK)-dependent process, where it acts upstream of JNK in LE cells. Using a yeast two-hybrid screen, the unique *Drosophila* homolog of the downstream of kinase (Dok) family, Ddok, was identified by its ability to bind Shark SH2 domains in a tyrosine phosphorylation-dependent fashion. In cultured S2 embryonic cells, Ddok tyrosine phosphorylation is Src dependent; Shark associates with Ddok and Ddok localizes at the cell cortex, together with a portion of the Shark protein. The embryonic expression pattern of Ddok resembles the expression pattern of Shark. *Ddok* loss-of-function mutant (*Ddok*^{PG155}) germ-line clones possess DC defects, including the loss of JNK-dependent expression of *dpp* mRNA in LE cells, and decreased epidermal F-actin staining and LE actin cable formation. Epistatic analysis indicates that *Ddok* functions upstream of *shark* to activate JNK signaling during DC. Consistent with these observations, Ddok mutant embryos exhibit decreased levels of tyrosine phosphorylated Shark at the cell periphery of LE and epidermal cells. As there are six mammalian Dok family members that exhibit some functional redundancy, analysis of the regulation of DC by Ddok is expected to provide novel insights into the function of the Dok adapter proteins.

KEY WORDS: *Drosophila* embryogenesis, Morphogenesis, Shark tyrosine kinase, Dorsal closure, Dok, Jun kinase

INTRODUCTION

The synchronized movement of epithelial cell layers is an essential aspect of morphogenesis in the development of many animals (Martin and Wood, 2002). Embryonic dorsal closure (DC), which represents such an epithelial morphogenetic event in *Drosophila*, begins at stage 11 when, following germband retraction, a dorsal hole appears in the epidermis that is covered by an extra-embryonic epithelium known as the amnioserosa (Martinez-Arias, 1993). DC is the process in which the bilateral dorsal movement of the epidermis over the amnioserosa, followed by the dorsal suturing of the leading edge (LE) cells, leads to the complete enclosure of the embryonic viscera. This epidermal sheet movement is accomplished by the elongation of epidermal cells without their proliferation or the recruitment of any additional cells (Martinez-Arias, 1993). DC is a particularly well-characterized system for the elucidation of signaling pathways regulating the movement and fusion of epithelial sheets in development (Martin and Wood, 2002), and may constitute a useful model system for studies of wound healing (Jacinto et al., 2001).

Mutants in which the process of DC fails are characterized by the presence of a dorsal hole in the embryonic cuticle ('dorsal-open' or 'dorsal hole' phenotype) that is due to a failure of closure or suturing of the epidermal cells (reviewed by Noselli, 1998; Noselli and Agnes, 1999). Mutant identification has enabled multiple signal transduction pathways to be shown to participate in this process. These include the Jun amino-terminal kinase (JNK), Dpp and Wingless signaling pathways. In addition, the cell shape changes that occur during DC are regulated by the Rho family of small GTPases (reviewed by Harden, 2002). Finally, DC requires the involvement of genes encoding membrane proteins such as *canoe* and *myospheroid*, as well as genes encoding cytoskeletal proteins (Noselli and Agnes, 1999).

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Accepted 4 November 2005

Regulation of Dpp expression in LE cells by the JNK cascade is a central signaling pathway controlling DC. The LE cells, in turn, signal to more lateral cells in the epidermis through the Dpp receptor, inducing them to participate in DC (reviewed by Harden, 2002). Mutants in many members of the JNK pathway have been shown to influence DC and these mutants lead to a loss of DPP expression in the LE cells (Glise and Noselli, 1997; Stronach and Perrimon, 2002). These mutations can be rescued by expression of a constitutively activated form of Jun (previously known as c-Jun; Jra – FlyBase). This group of genes includes *misshapen* (*msn*), which encodes a germinal center kinase, *slipper* (*slpr*), which encodes a mixed lineage kinase (Stronach and Perrimon, 2002), *hep*, which encodes a MAP kinase kinase (Glise et al., 1995), *bsk*, which encodes *Drosophila* Jun kinase (Glise et al., 1995; Riesgo-Escovar et al., 1996), *Djun* (*Jra* – FlyBase) (Kockel et al., 1997; Nusslein-Volhard et al., 1984; Riesgo-Escovar and Hafen, 1997; Riesgo-Escovar et al., 1996; Sluss et al., 1996) and *kayak*, which encodes Dfos (Jurgens et al., 1984; Riesgo-Escovar and Hafen, 1997; Zeitlinger et al., 1997). Several other genes appear to play a role in the activation of JNK pathway signaling, but are not individually essential, in some cases because of functional redundancy (reviewed by Harden, 2002). For example, although neither *Src42A* nor *Tec29A* (*Btk29A* – FlyBase) Src kinase mutations individually exhibit a DC defect, *Src42A Tec29A* double mutants are DC defective, fail to exhibit LE cell Dpp expression, and are rescued by the expression of activated Jun (Tateno et al., 2000).

We have previously identified and characterized Shark (SH2 domain ankyrin repeat kinase (Ferrante et al., 1995), and have shown it to be an essential component of the JNK pathway acting during DC (Fernandez et al., 2000). Embryonic cuticles produced by *shark*^{1} germ-line clones exhibit a dorsal-open phenotype, and *shark*^{1} flies partially rescued by a *hs-shark* transgene produce a split thorax phenotype similar to that produced by insufficiency for other JNK pathway members (Glise et al., 1995; Riesgo-Escovar and Hafen, 1997; Zeitlinger et al., 1997). *shark*^{1} germline clones fail to express *dpp* in the LE cells and are rescued by the expression of activated Jun. These results and ectopic expression studies (Fernandez et al.,

2000) indicate that Shark functions upstream of JNK in the JNK signaling pathway regulating DC. To better understand the role of Shark, we performed a yeast two-hybrid screen for proteins that physically interact with the NH2-terminal regulatory regions. Here, we describe the characterization of one of the proteins that interacts with Shark, *Drosophila downsteam of kinase* (*Ddok*), which belongs to the Dok family of adaptor proteins. We show that *Ddok* is required for DC, functions upstream of Shark in the JNK pathway, and is required for the appropriate tyrosine phosphorylation and localization of Shark to the cell peripheries of LE and lateral epidermal cells.

MATERIALS AND METHODS

Yeast two-hybrid screen and plasmid constructions

A yeast two-hybrid screen was performed according to the manufacturer's instructions (Clontech, MATCHMAKER Kit). The cDNA encoding the NH_2-terminal region (aa 10-468) of Shark was subcloned into the pGilda vector at the *Sma*I and *Bam*HI sites to create pLexA-Shark encoding the LexA-Shark fusion protein bait. In addition, the cDNA encoding an activated Src-kinase domain was excised from the BTM116-Src vector (Keegan and Cooper, 1996; Lioubin et al., 1996) as a *Bam*HI fragment and subcloned into pLexA-Shark at the *Sac*I site to create pLexA-Shark/Src encoding activated Src kinase driven by the ADH1 promoter. Proteins from yeast transformed with this vector exhibited a high degree of tyrosine phosphorylation in anti-phosphotyrosine western blots (data not shown). A cDNA library from 0- to 21-hour-old *Drosophila* embryos (Clontech) cloned into pB42AD (Clontech) was used as the prey. These bait and prey plasmids were co-transformed into the *Saccharomyces cerevisiae* EGY48 (p8op-LacZ) host strain that carries *Leu2* and *lacZ* under the control of LexA operators. Approximately 5×10^6 co-transformants were screened on *Ura*$^-$, *His*$^-$, *Trp*$^-$, *Leu*$^-$ and X-gal plates. Seventeen independent transformants activated both the *lacZ* and *Leu2* reporter genes. The pB42AD plasmids were individually isolated from each of the yeast transformants and sequenced.

The cDNA encoding Ddok was amplified by PCR (Expand High Fidelity PCR System, Roche) from the FlyBase clone LD32155, using the following primers: forward, 5′CGGAATTCGCCACCATGGATGTTGA-AATACCT-3′; and reverse, 5′-GAGGAGTCTAGATTACTACTTATCG-TCGTCATCCTTGTAATCCACTCGCTTCGG-3′ (CO_2H-terminally tagged Ddok-Flag). The primers used for subcloning Shark were: forward, 5′-GAGCAGAAACTGATCAGCGAGGAGGACCTGAAATGGTAC-3′; and reverse, 5′-CATTTCAGGTCCTCCTCGCTGATCAGTTTCTGCTC-GTAC-3′ (NH_2-terminally tagged Myc-Shark). The PCR products were digested with *Eco*RI and *Xba*I (Ddok), or with *Not*I and *Xho*I (Shark), and cloned into the pMTA vector (Invitrogen).

Antibody production, immunostaining procedures and cuticle preparation

The region encoding the unique CO_2H-terminal (amino acid residues 241-622) of Ddok was cloned into pGEX-KG (Guan and Dixon, 1991) at the *Eco*RI and *Nco*I sites. The GST fusion protein was purified and injected into rabbits (Covance). Rabbit anti-Y927 Shark phosphopeptide antiserum was raised against a Shark phosphopeptide ARDPDY(PO_4)QNLPELVQTVHIC (pY927 peptide, amino acids 922-940) (coupled through Cys940 to Keyhole Limpet Hemocyanin). Anti-phosphoY927 antibody (anti-pY927) was purified from antibodies to unphosphorylated peptides in this antiserum by binding to a pY927 peptide column and eluting with 0.1 M glycine-HCl (pH 2.0) buffer. Anti-P-Tyr-100 was obtained from cell signaling. For the immunofluorescence studies, *Drosophila* Schneider (S2) cells were grown in Schneider Medium (Invitrogen) containing 10% fetal bovine serum (Invitrogen) on Polylysine (10 mg/ml)-coated coverslips. They were transfected (1×10^7 cells/ml, Ddok-Flag, Myc-Shark) with Effectine (QIAGEN), induced with $CuSO_4$ for 6 hours and grown for 48-72 hours in six-well plates. The cells were then washed with PBS and fixed with 4% paraformaldehyde for 20 minutes. Coverslips were blocked with 10% goat serum in PBS for 20 minutes, the cells incubated with primary (2 hours) and secondary (1 hour) antibodies with PBS washes before and after secondary antibody. Anti-Flag (Sigma) and anti-Myc (Invitrogen) antibodies were used at 1:500 dilutions. Immunofluorescence staining of embryos was performed using the anti-Ddok antiserum at 1:1000, the anti-LacZ antibody and the anti-Fasciclin III antibody (Developmental Studies Hybridoma Bank) at dilutions of 1:2000 and 1:40, respectively, and the anti-pY927 antibody at 1 μg/ml. Secondary antibodies for staining S2 cells and embryos were Alexa 488- or Alexa 594 (Molecular Probes)-conjugated mouse or rabbit IgG used at a dilution of 1:500. Embryos were fixed with 4% paraformaldehyde prior to immunostaining and devitellinized with methanol (or 80% cold ethanol for phalloidin staining). Cuticles were prepared as described previously (Wieschaus and Nusslein-Volhard, 1986), and photographed under bright and dark field using Ektachrome 160T film.

Immunoprecipitation and western blot

S2 cells were cultured in 100-mm diameter tissue culture dishes (Falcon) in Schneider medium containing 10% fetal calf serum at 22°C. For Src inhibitor studies, cells were treated with PP2 (10 μM, Calbiochem) for 1 hour. S2 cells transfected (Effectine, QIAGEN) with different plasmids (~10^7 cells/dish) were lysed in l ml of lysis buffer (10 mM Tris-HCl, 50 mM NaCl, 50 mM NaF, 30 mM sodium pyrophosphate, 5 μM $ZnCl_2$, 1 mM sodium orthovanadate, 1% NP-40, 1 mM Benzamidine, 10 μg/ml leupeptin and 5 μg/ml aprotinin, pH 7.2). The cell lysates were centrifuged (13,000 ***g***, 30 minutes, 4°C) and Sepharose G beads (20 μl, packed volume, Pharmacia) and Flag antibody (0.5 μg) were added to the supernatant, which was rotated at 4°C overnight. The beads were then washed with lysis buffer (four to five times, 500 μl), the immunoprecipitated proteins eluted with 10 μl of 3×SDS-PAGE loading buffer, subjected to SDS-PAGE, transferred to a Nylon 0.2 μm PVDF membrane and western blotted with primary antibodies at the following concentrations: RC20H Anti-Ptyr:HRP (Transduction Laboratories), 1:5000; anti-Myc monoclonal antibody (Invitrogen), 1:5000; rabbit anti-Shark antiserum (Fernandez et al., 2000), 1:1000; rabbit anti-Ddok,1:1000; anti-Y927 peptide antibody, 1 μg/ml; and anti-Flag monoclonal antibody (M2 monoclonal, Sigma), 1:5000.

In situ hybridization and RT-PCR

Whole-mount in situ hybridization was performed as described by Tautz and Pfeifle (Tautz and Pfeifle, 1989), with digoxigenin-labeled RNA probes prepared by in vitro transcription. The Ddok probe was 1241-1782 bp of cDNA sequence and the Dpp probe was the entire cDNA. RT-PCR was performed on RNA extracted from wild-type and *Ddok*^{PG155}*/Y* males with the following primers: forward, 5′-GGCTGGATATCTTAATGTGCCGAC-3′; reverse, 5′-GCGGCTCCTGAGTGATCTTCACA-3′ (the expected product size was 223 bp).

Genetic protocols

To eliminate both maternal and zygotic Ddok activity, germ-line clone (GLC) mutants of *Ddok*^{PG155} were generated as described previously (Chou et al., 1993). Virgin females of the genotype *y w Ddok*^{PG155} *FRT*^{101}*/FM7* were crossed to *ovo*^{D1} *FRT*^{101}*/Y; hs-Flp38* males. The larvae were heat shocked for 1 hour at 37°C on days 4, 5 and 6 AEL to induce recombination. GLC-containing females of the genotype *y w Ddok*^{PG155} *FRT*^{101}*/ovo*^{D1} *FRT*^{101}*; hs-Flp38* were then mated to wild-type males. Whenever it was necessary to distinguish GLC mutants from non-mutant flies a *ftz-lacZ* balancer was used. For the epistatic analyses, GLC virgin flies were crossed to males of the following genotypes: *hs-Src42A*$^{22.3}$*/hs-Src42A*$^{22.3}$ (Lu and Li, 1999), *hs-shark-10/hs-shark-10* (Fernandez et al., 2000), *hs-SEjun*^{asp}*/CyO* (Treier et al., 1995) and *puc*^{E69}*/TM3* (Glise and Noselli, 1997; Martin-Blanco et al., 1998). In crosses where heat shock was required, developmentally synchronized embryos were obtained and heat shocked for 30 minutes at 37°C during stages 9-10. For electron microscopy, a Jeol 6400 scanning electron microscope was used.

RESULTS

Ddok interacts with Shark

To identify proteins interacting with Shark, the NH_2-terminal non-catalytic region of Shark, comprising the NH_2-terminal Src homology 2 (SH2) domain, five ankyrin repeats, the carboxy-terminal SH2 domain and the proline-rich domain, was used as 'bait' in a yeast two-hybrid screen (Fields and Sternglanz, 1994).

Tyrosine phosphorylated residues mediate interaction with SH2 domains (Bradshaw and Waksman, 2002) and there is little tyrosine phosphorylation in *Saccharomyces cerevisiae*. To increase the probability of obtaining proteins whose interactions with Shark rely on the SH2 domains, we modified the yeast two-hybrid system by co-expressing the activated Src-kinase domain, which has been found to tyrosine phosphorylate library proteins in yeast (Keegan and Cooper, 1996; Lioubin et al., 1996). The bait plasmid was based on pGilda (Gimeno et al., 1996) and contained the LexA DNA-binding domain fused to the *shark* sequences, together with the DNA sequences encoding an activated Src-kinase domain (Lioubin et al., 1996), placed under the control of a constitutive promoter. The bait plasmid was co-transformed into yeast together with a *Drosophila* 'prey' library, in which cDNAs from 0- 21-hour-old embryos were fused to the B42 activation domain AD (Gyuris et al., 1993) (pB42AD; Clontech). Two out of the 17 clones whose interaction with the bait protein mediated LexA-dependent *lacZ* and *Leu2* reporter gene expression were shown to encode overlapping portions of a protein similar to the Downstream of kinase (Dok) family of proteins (Carpino et al., 1997; Di Cristofano et al., 1998; Ellis et al., 1990; Lemay et al., 2000; Yamanashi and Baltimore, 1997). The *Drosophila* Dok gene (*Ddok*) has been annotated in the *Drosophila* genome (Accession Number CG2079), and is predicted to encode a protein of 622 amino acids. When the screen was repeated using the same LexA-Shark-containing plasmid but lacking the Src kinase domain as bait, the positive clones obtained did not include Ddok sequences. Dok family proteins share a similar domain structure, comprising a Pleckstrin homology (PH) domain (Yan et al., 2002) and a phosphotyrosine-binding (PTB) domain (Lemmon, 2003), which are believed to confer association with the plasma membrane and with the tyrosine phosphorylated motif NPXpY, respectively (Yan et al., 2002; Lemmon, 2003). The predicted Ddok protein has a PH domain (amino acid residues 6-113; Fig. 1A) and a PTB domain (amino acid residues 138-234; Fig. 1B). The C-terminal tail of Ddok contains proline-rich stretches, which, in the mammalian Doks, are known to be docking sites for the Src homology 3 (SH3) domain-containing Src and Abl kinases (Master et al., 2003). In addition, Ddok is predicted to contain five putative sites for phosphorylation on tyrosine (Fig. 1C), which may serve as docking sites for SH2 domain-containing proteins. The PH and PTB domains are the regions exhibiting the greatest sequence similarity among the mammalian Doks, and between Ddok and the mammalian Doks (Fig. 1A,B). Ddok and the mammalian Doks are predicted to have arisen from a single primordial gene (Fig. 1D).

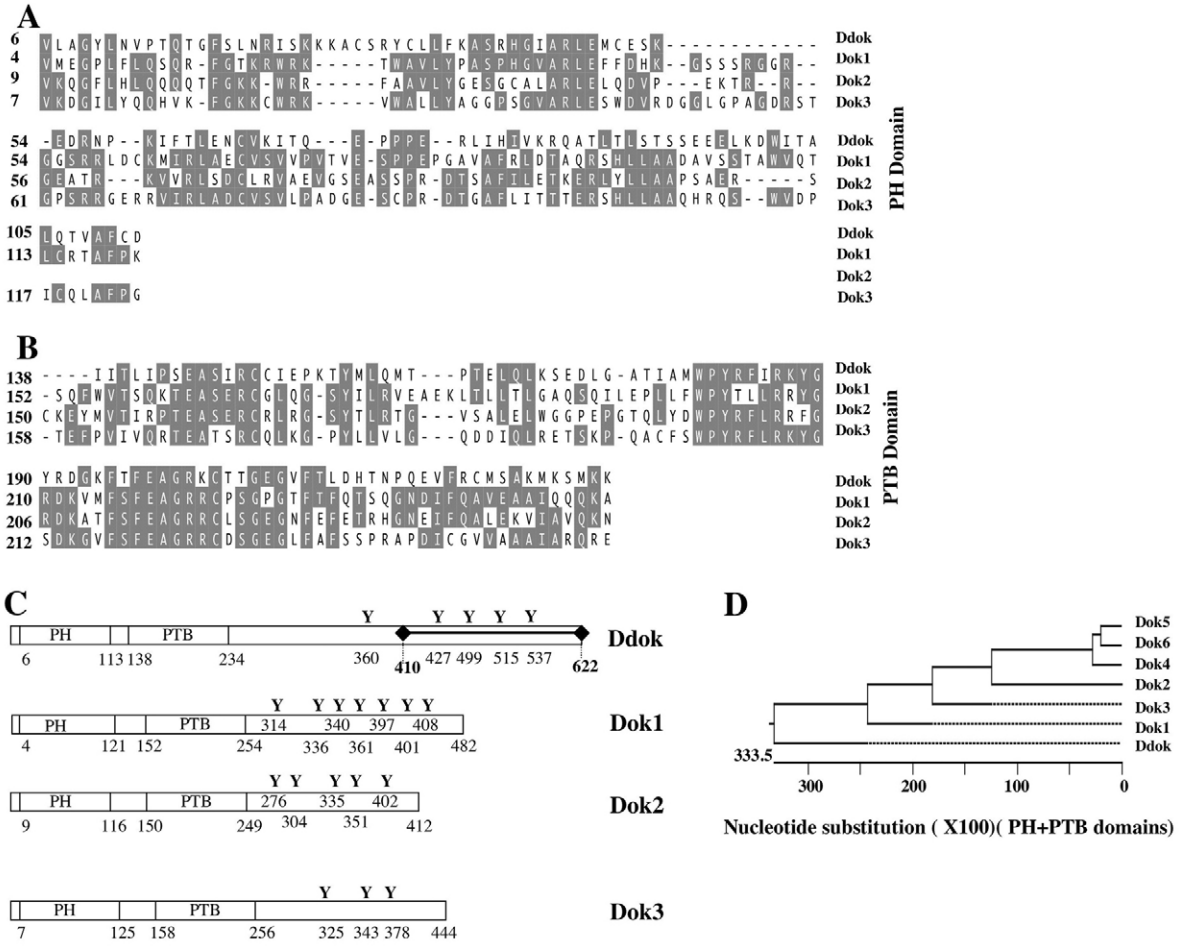

Fig. 1. *Drosophila* Dok sequences and their relationship to the mammalian Dok proteins. (**A**) Alignment of the Ddok PH domain with mammalian Dok1, Dok2 and Dok3. Identical amino acids are indicated by shading. (**B**) Alignment of the Ddok PTB domain with the PTB domains of mammalian Dok1-Dok3. (**C**) Schematic representations of Ddok and Dok1-Dok3, showing C-terminal tyrosines predicted to be phosphorylated by NetPhos. The line joining diamonds in Ddok shows the region of overlap of the Ddok clones obtained in the two-hybrid screen. (**D**) A phylogenetic tree of Ddok and the mammalian Dok nucleotide sequences compiled using the Clustal W method within the Lasergene Navigator program.

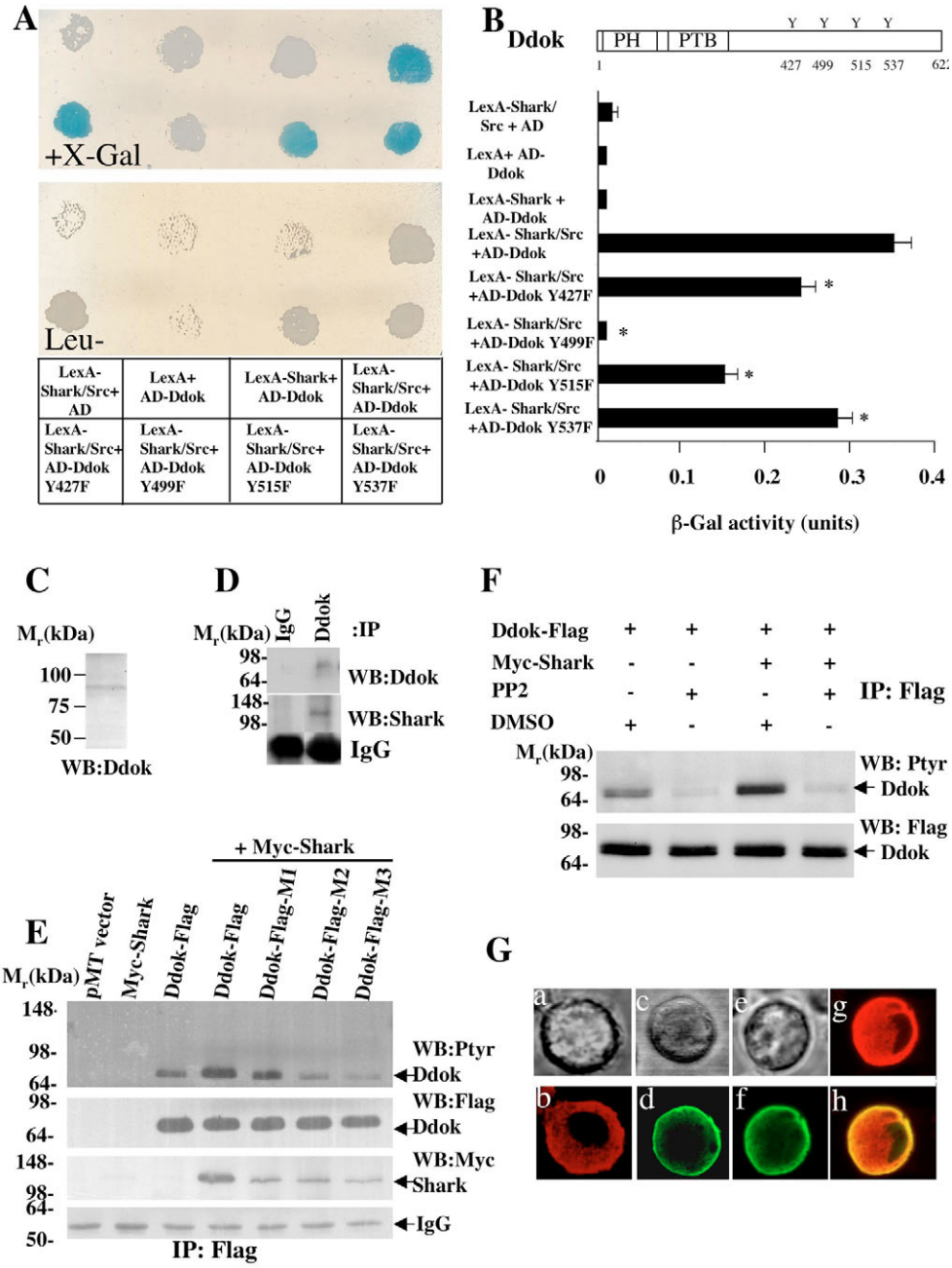

Fig. 2. Interaction of Shark and Ddok in yeast and S2 cells. (**A**) Dependence of the interaction between Shark and Ddok on tyrosine phosphorylation. LexA, the DNA-binding domain in the pGilda vector was fused to Shark in pGilda without activated Src (Lex A-Shark), to Shark in pGilda with activated Src (LexA-Shark/Src) or, as a control, to nothing (LexA). AD, the activation domain in the pB42AD vector was fused to either Ddok (AD-Ddok), DdokY→F mutations (AD-Ddok Y---F) or nothing (AD). Transformed yeast were plated on replica plates containing X-gal for screening β-galactosidase-positive blue colonies (upper panel) and Leu⁻ plates for screening Leu-positive colonies (lower panel). (**B**) Y427, Y499, Y515 and Y537 are involved in the tyrosine phosphorylation-dependent interaction of Ddok with Shark. The constructs used in A were used to perform the more sensitive β-galactosidase assays of yeast cell lysates (±s.e.m.; n=3; *P<0.05, Student's t-test, significantly different from LexA-Shark/Src+AD-Ddok). (**C**) Ddok antibody detects a single protein in a western blot of *Drosophila* embryo extract. (**D**) Endogenous Shark and Ddok are associated in S2 cells. (**E**) Dependence of Shark and Ddok association on Ddok tyrosine phosphorylation in co-transfected S2 cells. S2 cells were transfected with pMT vector alone, Myc-Shark alone, Ddok-Flag alone, both Myc-Shark and Ddok-Flag, or Myc-Shark with each of the following Ddok-Flag mutants individually: Ddok-M1 (Ddok Y499F,Y515F), Ddok-M2 (Ddok Y499F,Y515F,Y427F), or Ddok-M3 (Ddok Y499F,Y515F,Y427F,Y537F). Cell lysates were immunoprecipitated with Flag antibody and subjected to SDS-PAGE and western blotting with the indicated antibodies. (**F**) Inhibition of Ddok tyrosine phosphorylation in S2 cells by the Src inhibitor, PP2. Ddok-Flag or Ddok-Flag+Shark transfected cells were incubated in medium containing 10 μm PP2 or solvent (DMSO) for 1 hour prior to the immunoprecipitation of cell lysates with Flag antibody. (**G**) Ddok and Shark are colocalized at the cell cortex. S2 cells were transfected with Myc-Shark alone (a,b), Ddok-Flag alone (c,d), or co-transfected with Myc-Shark and Ddok-Flag (e-h), immunofluorescently stained for Myc (red; b,g,h) and Flag (green; d,f,h), and examined by confocal microscopy. The merged panel (h) shows the colocalization (yellow).

The interaction between Shark and Ddok depends on tyrosine phosphorylation of Ddok

To determine the requirement for tyrosine phosphorylation of Ddok in its interaction with Shark in the yeast two-hybrid system, we examined the interaction in the presence and absence of an activated Src kinase. The interaction between the Shark NH_2-terminal region and Ddok was shown to be dependent on the presence of activated Src (Fig. 2A,B). To determine which tyrosines contained within Ddok were crucial for the interaction with the Shark SH2 domains, we examined the interaction between the Shark NH_2-terminal region and several Ddok CO_2H-terminal domain mutants possessing different tyrosine-to-phenylalanine point mutations. In a background of yeast expressing activated Src, Ddok Y427F, Y499F, Y515F and Y537F mutants were tested for their abilities to combine with LexA-Shark to grow on minimal medium plates lacking leucine, and to direct the expression of β-galactosidase, when grown on plates containing complex medium. Only the Ddok Y499F failed to interact with LexA-Shark in these assays (Fig. 2A). In a more sensitive liquid β-galactosidase assay, Ddok Y499F again failed to interact with LexA-Shark, but Ddok Y515F, Y427F and

Y537F also exhibited varying, decreased levels of interaction with the Shark NH_2-terminal regulatory domain (Fig. 2B), suggesting that multiple phosphotyrosines participate in binding.

By using large amounts of cell lysate and the Ddok-GST fusion protein antibody, which detected a single protein of the expected M_r (~74 kDa) in *Drosophila* embryo lysates (Fig. 2C), the association of Ddok with Shark could be detected in untransfected S2 cells (Fig. 2D). We therefore investigated the interaction between Ddok and Shark by expressing epitope-tagged versions of these proteins in S2 cells. Ddok-Flag, when overexpressed in S2 cells and immunoprecipitated with anti-Flag antibody, migrated as a single band on SDS-PAGE and was tyrosine phosphorylated (Fig. 2E, lane 3). When lysates from Myc-Shark and Ddok-Flag co-transfected cells were used, Shark (M_r ~116 kDa) was co-immunoprecipitated with Ddok, which exhibited increased tyrosine phosphorylation (Fig. 2E, lane 4). No tyrosine phosphorylation of Shark was detected (Fig. 2E, upper panel), suggesting that Shark itself was not activated by its association with Ddok. However, because Src42A and Src64B were both recently reported to associate with Ddok in a yeast two-hybrid analysis of *Drosophila* proteins (Giot et al., 2003), we examined the effect of the Src inhibitor PP2, which does not inhibit Shark tyrosine kinase activity (K. Hong, Y. G. Yeung and E.R.S., unpublished). Consistent with the involvement of Src kinases in the phosphorylation of Ddok, PP2 inhibited Ddok tyrosine phosphorylation in the presence or absence of transfected Shark (Fig. 2F).

We also investigated the role of the Ddok tyrosines shown to be required for the Src-kinase-dependent interaction in the two-hybrid system (Fig. 2B). In contrast to the interaction between Ddok and Shark fragments studied in the two-hybrid analysis, individual Ddok Y427, Y499, Y515 or Y537 mutations in full-length Ddok exhibited the same degree of association as unmutated Ddok (data not shown), whereas Ddok Y499, 515F double mutants showed a reduced association with Shark in S2 cells (Fig. 2E, lane 5). However, the interaction between Shark and Ddok was not reduced further by the introduction of additional mutations Y427F and Y537F (Fig. 2E, lanes 6,7). Thus results from both systems emphasize the importance of Ddok Y499 and Y515. The lack of effect of the individual Y mutants in the S2 cell system may reflect the presence of additional interactions due to the use of full-length constructs and a *Drosophila* system that is likely to be more permissive for the participation of third party proteins. As the sequence surrounding the Ddok Y499 and Y515 comprises an immunoreceptor tyrosine-based activation motif (ITAM) (Isakov, 1998), these data suggest that the Ddok ITAM plays a dominant, but not exclusive role in the interaction between Shark and Ddok in S2 cells. There was no interaction between Ddok and Shark lacking the SH2 domain-ankyrin repeats-SH2 domain regions (data not shown).

Myc-Shark expressed in S2 cells in the absence of Ddok was present mainly in the cytoplasm with some expression at the cell cortex (Fig. 2G, part b). By contrast, Ddok-Flag expressed in S2 cells localized primarily to the cell cortex with slight cytoplasmic expression (Fig. 2G, part d). When the two proteins were expressed together in S2 cells, there was no change in their distribution and their colocalization was restricted to the cell cortex (Fig. 2G, parts f-h).

Ddok expression pattern

Ddok protein is expressed at all stages of embryonic development, as demonstrated by staining with an antibody raised against the Ddok-GST fusion protein. Maternal expression of Ddok protein leads to strong expression in early stage embryos (see Fig. S1A,B in the supplementary material). Ddok is highly localized to the cortex of blastoderm embryos at the onset of cellularization (see Fig. S1B in the supplementary material). During germ band extension, it is expressed in the cephalic furrow and the proctodeum (see Fig. S1C in the supplementary material), and in the anterior and posterior midgut regions. By stage 13, Ddok protein is expressed in the epidermis, malphigian tubules and hindgut (see Fig. S1F). At later stages of development, it is expressed in midgut constrictions and the ventral nerve cord (data not shown). In parallel with the analysis of Ddok protein expression, the mRNA expression pattern was studied with an anti-sense RNA probe specific for the region of the Ddok gene encoding its C-terminal tail. The pattern of expression of the *Ddok* mRNA was similar to the protein expression pattern (Fig. S1G-L in the supplementary material). However, the abundance of the mRNA was observed to decline gradually after stage 13 of embryogenesis. The patterns of expression of the Ddok mRNA and protein resemble those of the Shark mRNA and protein (Fernandez et al., 2000).

A mutation in Ddok affects embryonic DC

The *Ddok* gene is located in region 7B1 on the X-chromosome. A screen for lethal mutations on the X-chromosome yielded an insertion of the P element transgene pGaw in the first intron of the *Ddok* gene, ~387 bp upstream of the second exon ($Ddok^{PG155}$) (Bourbon et al., 2002) (Fig. 3A). $Ddok^{PG155}$ is semilethal in hemizygous males and Δ2-3 transposase-mediated P-element-mediated allelic reversion led to the generation of lines in which males carrying the revertant chromosome were viable, indicating that the P-element insertion in $Ddok^{PG155}$ is the basis for lethality (Bourbon et al., 2002). $Ddok^{PG155}/Y$ males can also be rescued by overexpression of a Ddok transgene. The rescued flies are normal and fertile. Among the progeny of the cross $Ddok^{PG155}/FM7 \times FM7/Y$, 1-2% of the expected number of $Ddok^{PG155}/Y$ males survived to adulthood, and we considered these flies to be 'escapers' of the lethal phenotype. RT-PCR performed on the $Ddok^{PG155}$ hemizygous males failed to detect Ddok transcripts (Fig. 3B). The escapers exhibited four distinct phenotypes: the majority (~50%) exhibited a phenotype in which the wings are shriveled (Fig. 3C, part b,c). A similar wing phenotype of varying severity has also been reported in *hep* (*hemipterous*) mutants (Agnes et al., 1999). Another commonly observed phenotype is a split thorax phenotype (~30%) (Fig. 3D, part a,b), accompanied by the loss of bristles in the midline region of the thorax (Fig. 3D, part b). Both of these phenotypes are also associated with weak *hep* alleles (Glise et al., 1995), *kay* mutants that have been partially rescued through transgenic expression of the *kay* gene (Riesgo-Escovar and Hagen, 1997; Zeitlinger et al., 1997), and with partially rescued $Shark^{1}$ mutants (Fernandez et al., 2000). The other phenotypes displayed by $Ddok^{PG155}$ hemizygous flies include unilateral or bilateral loss of anterior orbital bristles (<10%) in the head region (Fig. 3D, part c,d), and irregularly placed bristles in the eyes (Fig. 3D, part e,f).

The high maternal expression of *Ddok* mRNA and protein, and the ability of embryos hemizygous for $Ddok^{PG155}$ to hatch and in some cases to survive to adulthood, suggested that maternally expressed Ddok could support the need for Ddok activity during embryogenesis. To eliminate the maternal *Ddok* contribution, germ-line clones (GLC) homozygous for $Ddok^{PG155}$ were generated (Chou and Perrimon, 1996). In cuticle preparations, approximately 50% of the embryos derived from these GLCs exhibited a severe dorsal open phenotype (Fig. 4A, part a,b; Table 1). Those progeny that did not die as embryos survived to adulthood, and all of them were female. This indicates that the paternally supplied X-chromosome, carrying the wild-type copy of Ddok, was capable of rescuing the GLC-

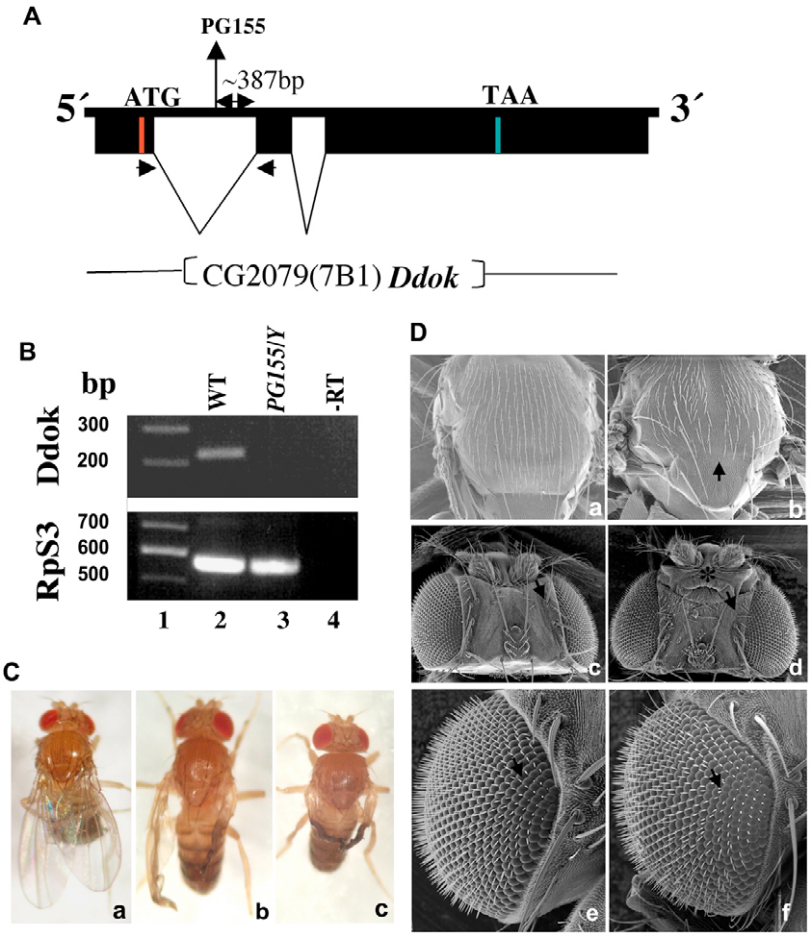

Fig. 3. *Ddok* gene organization and the phenotypes of *Ddok*^{PG155}*/Y* male 'escapers'. (**A**) Ddok gene organization, showing the position of the P-element insertion in the *Ddok*^{PG155} line. (**B**) RT-PCR with Ddok and RpS3 (control) primers. Lane 1, 100 bp DNA markers; lane 2, wild-type (WT) flies, with expected 223 bp product; lane 3, *Ddok*^{PG155}*/Y* flies, no detectable product; lane 4, no reverse transcriptase (–RT). (**C**) The wings of the male escapers are shriveled (b,c) compared with wild-type wings (a). (**D**) Defects (arrowed) in thorax (b), head (d) and eye (f) of the male escapers compared to wild-type thorax (a), head (c) and eye (e). Male escapers exhibit a split thorax phenotype (b) with loss of bristles at the dorsal midline (arrow). Occasionally, the escapers also show loss of the anterior orbital bristle in the head (d; asterisk indicates the normal developmental process) and irregularly placed bristles (f) in the eyes (arrows).

associated defect, and that the embryos that died with a dorsal open phenotype represented the male progeny of GLC-producing females and were both maternally and zygotically mutant for Ddok. Immunohistochemical staining with anti-Ddok antiserum supports the notion that the phenotypes associated with embryos derived from *Ddok*^{PG155} GLCs result from drastically reduced levels of Ddok protein. Although lateral epidermal cells of stage 13 wild-type embryos showed strong staining with Ddok specific antiserum (Fig. 4B, part a) at the cell periphery of the lateral epidermal cells and at the LE, similar staged embryos that were derived from *Ddok*^{PG155} GLCs did not express the Ddok protein, as revealed by immunohistochemical staining (Fig. 4B, part b). Wild-type embryos also showed a low level of staining of the aminoserosa.

The dorsal open phenotype observed for embryos lacking both maternal and zygotic Ddok expression is similar to that produced by GLC-derived mutants of JNK pathway genes, the expression of which is required in LE cells in order for DC to occur (reviewed by Fernandez et al., 2000). The DC defect in such embryos results from the failure of epidermal cells to stretch and elongate. Analysis of the epidermal cell morphology of both wild type and maternal/zygotic *Ddok*^{PG155} mutants by staining with anti-Fasciclin III antibodies revealed that, in contrast to the elongated lateral epidermal cells observed in wild type embryos (Fig. 4C, part a), the epithelial cells of the *Ddok*^{PG155} mutant embryos, like those of JNK pathway mutant embryos, are rounded (Fig. 4C, part b).

The expression of Dpp in LE cells, regulated through the JNK pathway, is observed as two lateral stripes, one on either side of stage 11 and 13 embryos (Fig. 4D, part a,c). Whole-mount in situ hybridization with a digoxigenin-labeled antisense RNA probe specific for *dpp* revealed an absence of *dpp* staining, specifically in the LE cells of stage 11 (Fig. 4D, part b) and stage 13 (Fig. 4D, part d) *Ddok*^{PG155} GLC mutant embryos, maternally and zygotically mutant for *Ddok*^{PG155}. Loss of Dpp expression in the LE cells of *Ddok*^{PG155} mutant embryos, together with their dorsal-open phenotype, indicates that Ddok is a component of the JNK signaling contributing to the process of DC.

The effects of the *Ddok*^{PG155} mutation on DC was further investigated by F-actin staining of epidermis and amnioserosa of *Ddok*^{PG155}*/Y* and *Ddok*^{PG155} GLC embryos with phalloidin at 10 hours and 12 hours AEL (Fig. 5). In *Ddok*^{PG155}*/Y* embryos, the normal intensity and pattern of actin distribution was preserved. However, the actin cable in the LE cells exhibited minor kinking and regional loss of staining in parts (Fig. 5B), compared with wild-type embryos (Fig. 5A). By contrast, despite initially stretching prior to the failure of DC (Fig. 5C, part a), Ddok GLCs showed decreased F-actin staining of all cells and a severe unevenness of the LE, with a loss of staining of the actin cable and of actin protrusions at the LE (Fig. 5C, part a,b).

Ddok is an upstream component of the JNK signaling pathway

The phenotype of *Ddok*^{PG155} GLC mutant embryos indicates that Ddok functions in the JNK pathway. To determine whether Ddok acts upstream or downstream of JNK in the pathway, we tested whether constitutively active Jun (*hs-SEjun*^{asp}) (Treier et al., 1995) could rescue the DC phenotype of *Ddok*^{PG155} GLC-derived

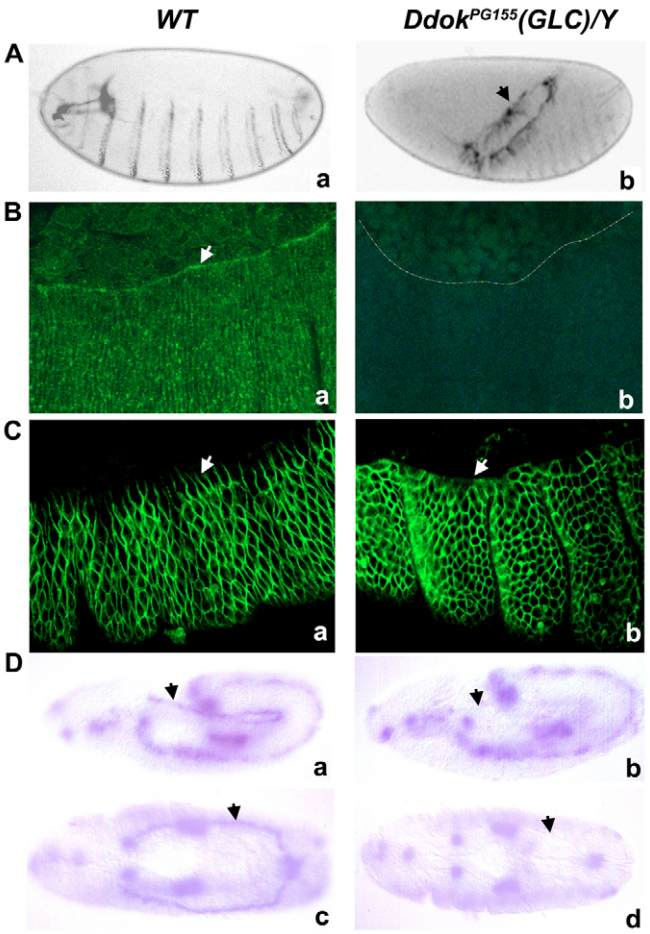

Fig. 4. Characterization of *Ddok*^{PG155} mutant embryos. (**A**) Bright-field photographs of cuticle preparations of wild-type and *Ddok*^{PG155}(GLC) embryos. *Ddok*^{PG155}(GLC) cuticles exhibit a severe dorsal open phenotype with large anterior holes (arrow). (**B**) Ddok immunofluorescent antibody staining of lateral epidermal cells of stage 13 embryos. Arrow and hatched line indicate LE. (**C**) Anti-Fasciclin III antibody staining of epidermal cells of stage 12 embryos. (**D**) In situ hybridization to *dpp* mRNA in *Ddok*^{PG155} GLC reveals a loss of *dpp* mRNA specifically from the LE cells (lateral stripes, arrows) in lateral views of stage 11 (a,b), and in dorsal views of stage 13 (c,d), embryos. *dpp* mRNA staining in other structures of the stage 11 and stage 13 mutant embryos is indistinguishable from that in wild-type embryos.

embryos. Although 48% of GLC-derived embryos exhibited a dorsal open phenotype, only 31% of embryos from GLC-producing females crossed to *hs-SEjun*^{asp} shared this phenotype (Table 1). The percentage of rescue observed (19%) is significant, as *hs-SEjun*^{asp} fathers are heterozygous for the transgene and only 25% of the unhatched embryos are expected to contain it. Cuticles of these embryos exhibited a less severe DC defect (small anterior holes) than the dorsal open phenotype of the unrescued embryos (Fig. 6B). These rescued embryos die at late embryonic stage and do not hatch to larvae.

The phosphatase Puckered negatively regulates JNK pathway signaling by dephosphorylating JNK, and loss of one copy of *puc* augments JNK pathway signaling (Glise and Noselli, 1997; Martin-Blanco et al., 1998). Males heterozygous for the *puc*^{E69} mutation (Glise and Noselli, 1997; Martin-Blanco et al., 1998) were crossed to *Ddok*^{PG155} GLC females to obtain *Ddok*^{PG155} male embryos heterozygous for *puc*^{E69}. In these crosses, we also observed a decrease in the number of GLC-derived embryos that were DC defective, from 48% to 29% of progeny (Table 1), and embryos exhibiting rescue possessed small holes in the cuticle (Fig. 6C), consistent with regulation of JNK signaling by Ddok that is upstream of *bsk*. To investigate the epistatic relationship between *shark* and *Ddok*, we similarly tested whether the *Ddok*^{PG155} GLC DC defect could be rescued by overexpression of Shark. Examination of the cuticles of embryos from the cross of *Ddok*^{PG155} GLC flies with males homozygous for *hs-shark-10* (Fernandez et al., 2000) (Fig. 6D), but not *hs-shark-K698R* (Fig. 6E), revealed a reduction in the percentage of embryos that were DC defective, from 48% to 10% (Table 1), and embryos exhibiting rescue possessed small anterior holes (Fig. 6D). These results suggest that Shark acts downstream of Ddok in the JNK pathway, and that Shark kinase activity is required for signaling.

Src family kinases act upstream of Syk family kinases in mammalian cells. As Shark belongs to the Syk family, we also examined the epistatic relationship between Src42A and Ddok by crossing *Ddok*^{PG155} GLC flies to male flies homozygous for *hs-Src42A*$^{22.3}$ (Lu and Li, 1999) (Fig. 6E). The percentage of DC defective embryos from this cross was not significantly altered (from 48% to 44%) by expression of Src42A, indicating that Src is not capable of rescuing the Ddok mutant phenotype. Furthermore, an activated form of *Src* (*UAS-Src42A.CA*) (Tateno et al., 2000) driven by *hs-Gal4* failed to rescue *Ddok*^{PG155} mutants.

Decreased expression of tyrosine phosphorylated Shark at the cell periphery in *Ddok* mutant embryos

As Ddok is expressed at the cell peripheries of LE, lateral epidermal and S2 cells, and overexpression of Shark rescues DC in *Ddok* mutant embryos, one mechanism of action of Ddok could be to localize Shark for activation. However, there was no discernable difference between wild-type and Ddok mutant embryos stained with anti-Shark antiserum (data not shown). We therefore prepared a phosphopeptide antibody (anti-pY927) to a Shark phosopeptide containing Y927, which is phosphorylated when Shark is activated in S2 cells (K. Hong, Y. G. Yeung and E.R.S., unpublished). In contrast to the anti-phosphotyrosine antibody, which recognizes many bands in embryo extract western blots (Fig. 7A, lane 2), anti-pY927 recognizes only a single band (Fig. 7A, lane 1) with the M_r of Shark (~116 kDa); this recognition is lost on prior phosphatase treatment of the blot (Fig. 7B), indicating that recognition by anti-pY927 is dependent on phosphorylation. Staining of wild-type embryos with anti-pY927 revealed that tyrosine phosphorylated Shark was localized to the cell peripheries of LE and lateral epidermal cells (Fig. 7C). By contrast, this intense localization of pY927 Shark was substantially reduced in *Ddok*^{PG155} GLC embryos (Fig. 7D). These data indicate the involvement of Ddok in the localization and activation of Shark during DC.

DISCUSSION

We have previously shown that the activity of the non-receptor tyrosine kinase Shark is essential for *Drosophila* embryonic DC (Fernandez et al., 2000). Using a two-hybrid approach we have now identified tyrosine phosphorylated Ddok to be a Shark-interacting partner, and we have confirmed this interaction in *Drosophila* cells in tissue culture. The similar embryonic expression patterns of Shark and Ddok are consistent with their functional interaction, which is supported by evidence that Ddok regulates the JNK signaling pathway. A mutation in Ddok exhibits phenotypes associated with

Table 1. Quantitation of the dorsal open phenotype in epistatic analysis

Cross	Expected if fully rescued		Observed			Total
	% DC	% DO	% DC	% DO	% SH	*n*
Ddok^{PG155}/Ddok^{PG155}(GLC)×+/*Y*	50	50	52	48	0	486
Ddok^{PG155}/Ddok^{PG155}(GLC)×+/*Y; hs-jun^{asp}*/+; +/+ (+hs)	75	25	50	31	19	100
Ddok^{PG155}/Ddok^{PG155}(GLC)×+/*Y*; +/+; *puc^{E69}*/+	75	25	50	29	21	601
Ddok^{PG155}/Ddok^{PG155}(GLC)×+/*Y*; +/+; *hs-shark-10/hs-shark-10* (+hs)	100	0	50	10	40	392
Ddok^{PG155}/Ddok^{PG155}(GLC)×+/*Y*; +/+; *hs-shark-K698R/hs-shark-K698R* (+hs)	100	0	53	47	0	400
Ddok^{PG155}/Ddok^{PG155}(GLC)×+/*Y*; +/+; *hs-Src42A$^{22.3}$/hs-Src42A$^{22.3}$* (+hs)	100	0	56	44	0	184

Embryos were collected and aged for 24 hours after egg lay. In all heat-shock (hs) experiments, 5- to 8-hour staged embryos were heat shocked at 37°C for a period of 30 minutes for the induction of the transgene. The total number of embryos and hatched larvae (*n*) was determined by counting in Hoyers solution. Cuticles were prepared from unhatched embryos, and the number of embryos with dorsal closed (DC), dorsal open (DO) and small hole (SH) cuticles enumerated. The % DC group includes hatched larvae as well as embryos with DC articles.

other JNK pathway members: split thorax and shriveled wings in adults, and DC defects in embryos. At the cellular level, the failure of JNK signaling in embryos lacking Ddok results in an absence of Dpp expression in LE cells. Consequently, epidermal cells in these mutants fail to stretch and elongate as they should during DC. The rescue of the DC defect of *Ddok^{PG155}* GLC mutant embryos by heat shock-mediated overexpression of an activated form of Jun (*hs-cJun^{asp}*), and by a single loss-of-function allele of *puc* (*puc^{E69}*), a phosphatase that negatively regulates JNK, indicates that Ddok acts upstream of JNK in the pathway. Ddok also appears to act upstream of Shark, as heat shock-mediated overexpression of Shark (*hs-shark-10*) was capable of rescuing the *Ddok* mutant phenotype.

The interaction between the Ddok C-terminal and Shark N-terminal region was shown to be dependent on tyrosine phosphorylation of Ddok by activated Src, and to be reduced by mutation of each of four Ddok tyrosines to phenylalanine. Co-expression studies in S2 cells showed that full-length Shark associates with full-length Ddok, that Ddok is tyrosine phosphorylated and that Ddok tyrosine phosphorylation, in the presence or absence of Shark, is Src dependent. Two Ddok tyrosines, Y499 and Y515, are present within an ITAM consensus sequence. ITAMs consist of two repeats of conserved sequence (Tyr-X-X-Leu/Ile) spaced by six to eight residues (Isakov, 1998) and, when the conserved tyrosines are phosphorylated, serve as binding sites for SH2 domain-containing proteins, including Syk family members. The association of Shark with tyrosine phosphorylated Ddok was substantially decreased in the Ddok Y499, 515F double mutant, implying that association of Ddok and Shark is, in part, mediated by the ITAM involving these Ddok phosphotyrosines.

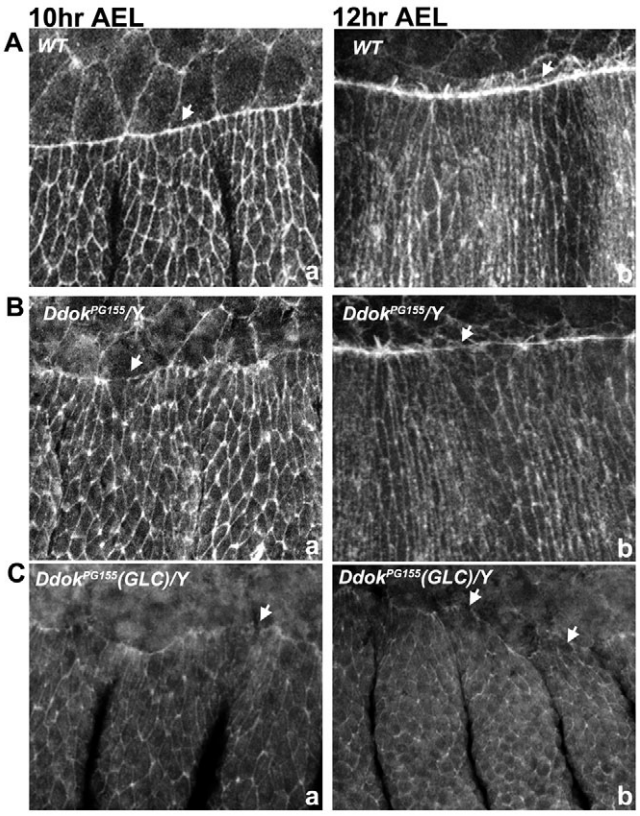

Fig. 5. Phalloidin staining of F-actin. Phalloidin staining of F-actin in (**A**) wild-type, (**B**) *Ddok^{PG155}/Y* and (**C**) *Ddok^{PG155}*(GLC) embryos at 10 and 12 hours after egg laying. Arrows indicate leading edge.

The major site of colocalization of Ddok and Shark in S2 cells is the cell cortex (Fig. 2F); Ddok and pY927Shark are localized in the epidermis (Fig. 7). Ddok possesses a PH domain, which, in other proteins, has been shown to bind to phospholipids in the plasma membrane (Lemmon, 2003). Studies on mammalian Dok1 plasma membrane recruitment and tyrosine phosphorylation suggest that activation of PI 3-kinase promotes the binding of the Dok1 PH domain to phosphoinositides and Dok1 recruitment to the plasma membrane where it is phosphorylated by Src, Tec and/or Abl family kinases (Liang et al., 2002; Nelms et al., 1998; Songyang et al., 2001; Suzu et al., 2000; Woodring et al., 2004; Zhao et al., 2001). The cell cortical localization of Ddok (Fig. 2F) and its ability to associate with Src kinases (Giot et al., 2003) suggest that it is positioned to respond to Src signaling. By contrast, Shark is expressed throughout the cytoplasm (Fig. 2F). During DC, increased activation of Src by appropriate upstream signals could increase Ddok tyrosine phosphorylation, resulting in increased localization of Shark to the cell cortex, where it becomes tyrosine phosphorylated and activated. Consistent with this suggestion, pY927 Shark is localized to the cortex in wild-type embryos, and this localization is substantially reduced in *Ddok^{PG155}* GLC embryos. The rescue of the *Ddok^{PG155}* DC defect by overexpression of Shark is easily understood in terms of this cell cortex-localizing function of Ddok, as overexpression of Shark may obviate the need for its directed localization.

Relevant to the synergism between Src and Shark in DC postulated above, Src42A and Shark appear to function together downstream of *bullwinkle* in stretch cells to regulate dorsal appendage cell movement in a JNK-independent fashion (Tran and Berg, 2003). Synergism between Shark and Ddok may take place in stretch cells, where overexpression of Shark suppressed the *bullwinkle*-mutant dorsal appendage phenotype. A similar synergism may take place during cardiogenesis, which involves Ddok regulation (Kim et al., 2004).

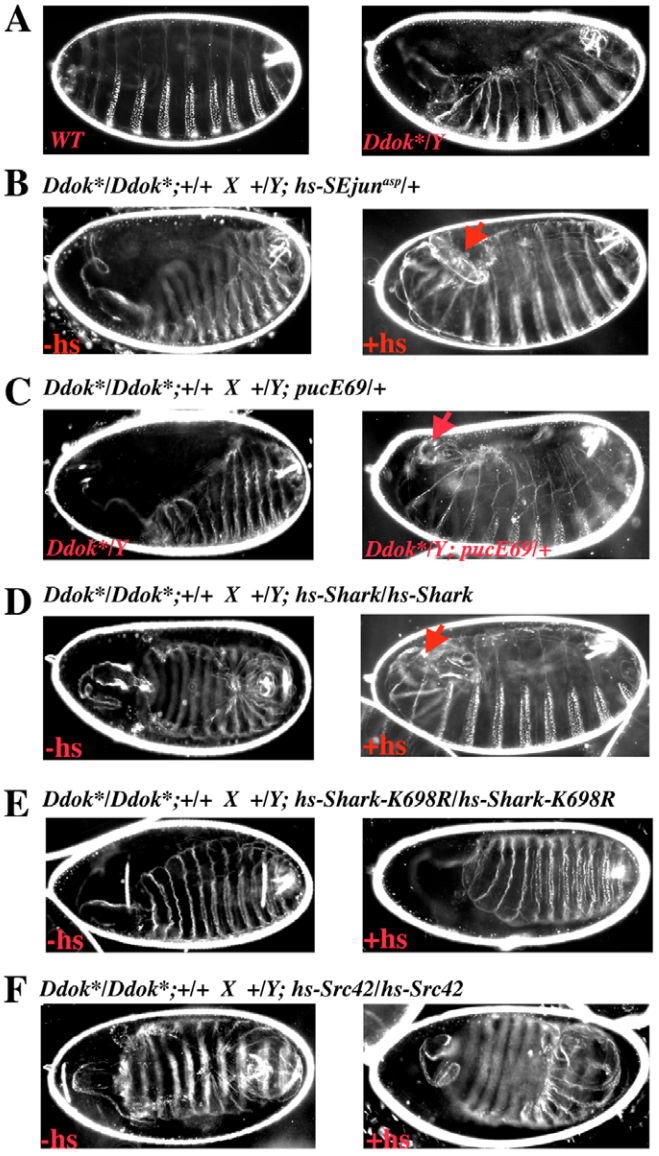

Fig. 6. Epistatic analysis of *Ddok* and components of the JNK signaling pathway. *Ddok*^{PG155} GLC flies were crossed to males bearing JNK pathway transgenes or mutations. Embryos bearing heat-shock transgenes were heat shocked for 30 minutes at 37°C at stage 9-11, and then kept at 25°C. Cuticle preparations of (**A**) wild-type embryos and *Ddok*^{PG155} mutant (*Ddok**) embryos, and (**B-F**) *Ddok*^{PG155} mutant embryos (right), (B) expressing a constitutively activated form of Jun (*hs-SEjun*^{asp}), (C) heterozygous for *puc*^{E69}, (D) overexpressing Shark (*hs-shark-10*), (E) overexpressing kinase-dead Shark (*hs-shark-10-K698R*) and (F) overexpressing Src42A (*hs-Src42A*$^{22.3}$), together with cuticles of control, DC-defective, *Ddok*^{PG155}*/Y* embryos (left). +hs, heat shocked; –hs, no heat shock. Arrows indicate partial rescue of the dorsal open phenotype.

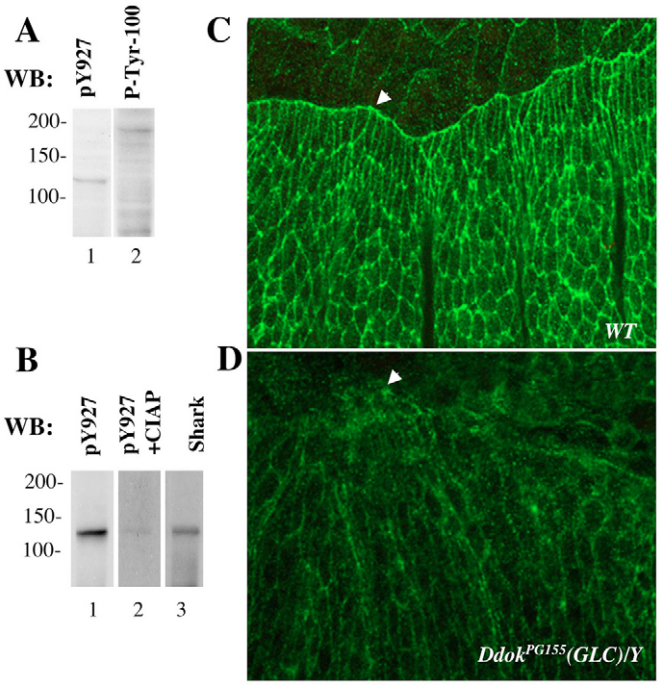

Fig. 7. Expression of tyrosine phosphorylated Shark at the periphery of epidermal cells is decreased in *Ddok* mutant embryos, during DC. (**A**,**B**) Embryo extract western blotted with (A) anti-pY927 and anti-phosphotyrosine (anti-P-Tyr-100) antibodies, and (B) anti-pY927, before (lane 1) and after (lane 2) phosphatase (CIAP) treatment of membrane, and with anti-Shark antiserum (lane 3). (**C**,**D**) Staining of wild-type and *Ddok*^{PG155} mutant embryos with anti-pY927 Shark peptide antibody. Arrowheads indicate the LE.

The other Syk family tyrosine kinases, Syk and the mammalian ZAP-70, both of which are predominantly localized to the cell cortical region of the T-cell (Huby et al., 1997), play important roles in immunoreceptor signaling (Chu et al., 1998; Latour and Veillette, 2001). In this study, we show that Ddok is required for the localization of Shark to the membrane, where it is tyrosine phosphorylated and probably activated. As Ddok functions to localize Shark to the plasma membrane, it is important to determine the mechanism by which Shark is activated. Given the established role of the Src family kinases Src42A and Tec29 in DC (Tateno et al., 2000), the Src dependence of Ddok tyrosine phosphorylation in S2 cells, the failure of *Src42A*$^{22.3}$ and *Src42A.CA* to rescue the DC defect of Ddok-deficient embryos, the association of Src42A and Src64B with Ddok (Giot et al., 2003), and the fact that Shark is a member of the Syk family of tyrosine kinases, activation of which is mediated by the Src kinases (Chan et al., 1994), the involvement of the Src kinases in the direct activation of Shark is an intriguing possibility.

The mammalian Dok family of proteins comprises six members: Dok1 (p62^{Dok}) (Ellis et al., 1990; Carpino et al., 1997; Yamanashi and Baltimore, 1997), Dok2 (or Dok-R/FRIP) (Di Cristofano et al., 1998; Jones and Dumont, 1998; Nelms et al., 1998), Dok3 (or Dok-L) (Cong et al., 1999; Lemay et al., 2000), Dok4, Dok5 (Grimm et al., 2001) and Dok6 (Crowder et al., 2004). Mammalian Doks have been shown to enhance (Hosooka et al., 2001) and to inhibit (Di Cristofano et al., 2001; Liang et al., 2002; Nelms et al., 1998; Songyang et al., 2001; Suzu et al., 2000) cell proliferation in several cell types. Relevant to our observation of the involvement of Ddok in DC, a morphogenetic process involving considerable cell shape changes, Dok1 has also been shown to activate cell spreading (Hosooka et al., 2001; Woodring et al., 2004) and insulin receptor-dependent cell migration (Noguchi et al., 1999), and Dok2 is involved in the Tie2-dependent migration of epithelial cells (Jones et al., 2003). However, the level of their involvement in the regulation of cell migration and growth in cultured cells in vivo remains unclear. Dok1$^{-/-}$ mice appear

normal, are fertile and live at least 1 year (Yamanashi et al., 2000). Similarly, Dok1$^{-/-}$; Dok2$^{-/-}$ double mutants are viable, fertile and born in appropriate Mendelian proportions (Niki et al., 2004; Yasuda et al., 2004). These mice develop myeloproliferative disease at ~1 year of age. As the expression patterns of the mammalian Dok proteins overlap, it is possible that they act redundantly, and that it will be necessary to eliminate the function of several Dok genes in order to obtain stronger phenotypic effects. Considerable insight into the function of Dok proteins may be gained from studies of Ddok, as it is the unique member of this family in *Drosophila*.

This work was supported by National Institutes of Health grants GM 55293 and CA26504 (to E.R.S.), and GM 52761 (to D.S.), and by the Albert Einstein Cancer Center (grant P30-13330; to E.R.S.). We thank L. R. Rohrschneider for the BTM116-Src vector and advice, N. Perrimon for providing us with *puc*^{E69} lines, H. Bourbon for the *Ddok*^{PG155} line, X. Lu for the *hs-Src42A*$^{22.3}$ line, K. Hong for the Shark amino-terminal deletion construct, Dr Y.-G. Yeung for advice, Ranu Basu for help and the Bloomington Stock Center for the providing us with several fly lines.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/2/217/DC1

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