**Dcdd42 acts in TGF-β signaling during Drosophila morphogenesis: distinct roles for the Drac1/JNK and Dcdd42/TGF-β cascades in cytoskeletal regulation**

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**SUMMARY**

During *Drosophila* embryogenesis the two halves of the lateral epidermis migrate dorsally over a surface of flattened cells, the amnioserosa, and meet at the dorsal midline in order to form the continuous sheet of the larval epidermis. During this process of epithelial migration, known as dorsal closure, signaling from a Jun-amino-terminal-kinase cascade causes the production of the secreted transforming-growth-factor-beta-like ligand, Decapentaplegic. Binding of Decapentaplegic to the putative transforming-growth-factor-beta-like receptors Thickveins and Punt activates a transforming-growth-factor-beta-like pathway that is also required for dorsal closure. Mutations in genes involved in either the Jun-amino-terminal-kinase cascade or the transforming-growth-factor-beta-like signaling pathway can disrupt dorsal closure. Our findings show that although these pathways are linked they are not equivalent in function. Signaling by the Jun-amino-terminal-kinase cascade may be initiated by the small Ras-like GTPase Drac1 and acts to assemble the cytoskeleton and specify the identity of the first row of cells of the epidermis prior to the onset of dorsal closure. Signaling in the transforming-growth-factor-beta-like pathway is mediated by Dcdd42, and acts during the closure process to control the mechanics of the migration process, most likely via its putative effector kinase DPAK.

Key words: *Drosophila*, Rac, Cdc42, JNK, TGF-β, DPAK, Small GTP-binding protein, Dorsal closure, Cytoskeleton, Morphogenesis

**INTRODUCTION**

Movement of the epidermis during the process of dorsal closure (DC) in *Drosophila*, is a good model system for the study of the regulation of cell migration (reviewed by Knust, 1997; Noselli, 1998; Martin-Blanco, 1997). DC is a morphogenetic process in which the two large sheets of lateral embryonic epidermis elongate along their dorso-ventral axis until they meet in the dorsal midline to form the continuous larval epidermis (Campos-Ortega and Hartenstein, 1985). An initial indication of the involvement of the cytoskeleton during DC was shown by Young et al. (1993) who reported accumulations of filamentous actin (F-actin) and non-muscle myosin (hereafter referred to as ‘myosin’) along the dorsal tip, or leading edge (LE), of the dorsal most row of epidermal cells during DC. They showed that myosin is required for DC by analysis of mutations in zipper, the gene encoding the non-muscle myosin heavy chain, and proposed a model by which the F-actin and myosin at the LE form a contractile apparatus to drive DC.

Members of the p21-Rho subfamily of Ras related small GTPases (comprised of the Rac, Cdc42, and Rho proteins) and their interacting molecules have been implicated in regulation of the cytoskeleton in many organisms (reviewed by Van Aelst and D’Souza-Schorey, 1997). Previously, we demonstrated that, in *Drosophila*, Drac1 is involved in localising F-actin and myosin to the LE (Harden et al., 1995), and that a *Drosophila* homologue, DPAK, of a downstream effector kinase Rac and Cdc42 (Manser et al., 1994, 1997), PAK (p21 activated kinase), is specifically upregulated at the LE during DC, reaching particularly high levels in cells at the segment borders (Harden et al., 1996). Subsequently, we have shown how the *Drosophila* Rho subfamily GTPases Drac1, Dcdd42, and DrhoA can each uniquely regulate the epidermal cytoskeleton to bring about the migration of the epidermis during DC (Harden et al., 1999). Drac1 and, to a lesser extent, Dcdd42 are involved in the initial assembly and/or maintenance of the LE cytoskeleton. Dcdd42 also has a role in regulating both the levels of DPAK at the LE and the mechanics of the DC process. DrhoA has a role in maintenance of the cytoskeleton specifically in LE cells flanking the segment borders during DC.

Cloning of a number of genes corresponding to DC mutants...
has led to the identification of two distinct signaling cascades operating during DC, a Jun-amino-terminal-kinase (JNK) cascade and a transforming-growth-factor-beta (TGF-ß) pathway. JNK cascades, also known as stress activated protein kinase (SAPK) cascades, consist of a set of sequentially activated kinases closely related to the extracellular regulated protein kinases (ERK) that transmit cytoplasmic signals to the nucleus (reviewed by Wilkinson and Millar, 1998). TGF-ß molecules are members of a large family of secreted growth factors that have been implicated in a wide variety of biological processes (reviewed by Kingsley, 1994; Wall and Hogan, 1994). Specific hetero-dimeric receptors that bind the TGF-ß ligands, and transmit signals to the cytoplasm via their intracellular kinase domains, have also been identified (reviewed by Massague, 1996). A new family of molecules, SMADs, that can regulate transcription, participate in signaling downstream of the TGF-ß receptors and have been characterised in many biological systems (reviewed by Derynck and Zhang, 1996; Wrana and Attisano, 1996; Massague et al., 1997). Intracellular MAPK/JNK-cascade-like kinases (Yamaguchi et al., 1995; Moriguchi et al., 1996; Atti et al., 1997a; Wang et al., 1997) and some of the small GTPases (Atti et al., 1997b; Mucsi et al., 1996) can also affect signaling downstream of TGF-ß receptors.

Involvement of a TGF-ß signaling pathway operating during DC3, was demonstrated by showing that mutations in the genes punt (put) (Childs et al., 1993; Ruberte et al., 1995; Letsou et al., 1995) and thick veins (tkv) (Penton et al., 1994; Nellen et al., 1994; Brummel et al., 1994; Affolter et al., 1994), which encode type-II and type-I TGF-ß receptors respectively, cause DC defects. Subsequent cloning and genetic interaction experiments with genes corresponding to the mutants pannier (pnr) (a GATA1-like transcription factor, Ramain et al., 1993; Affolter et al., 1994; Greider et al., 1995; Heitzler et al., 1996) and schnurri (shn) (a Zinc-finger DNA binding protein, Arora et al., 1995; Greider et al., 1995; Staehling-Hampton et al., 1995), originally isolated as mutants defective in DC, have illustrated involvement of a putative nuclear component to signaling downstream of the TGF-ß receptors Punt and Tkv. Recently the gene encoding a Drosophila SMAD, Mothers-Against-Dpp (Mad), has also been implicated in DC (Hudson et al., 1998).

Secreted TGF-ß family member, Decapentaplegic (Dpp), produced in the LE cells has been postulated to bind to, and initiate TGF-ß signaling via heterodimers of Punt and Tkv receptors in the leading edge cells. Subsequent cloning and characterisation of the genes corresponding to the DC mutants hemipterous (JNKK) (Glise et al., 1995; Glise and Noselli, 1997), basket (JNK) (Riesgo-Escovar et al., 1996; Riesgo-Escovar and Hafen, 1997a; Sluss et al., 1996; Sluss and Davis, 1997), l(2)IA109 (Djun) (Hou et al., 1997; Kockel et al., 1997) and kayak (Dfos) (Riesgo-Escovar and Hafen, 1997b; Zeitlinger et al., 1997) have identified a novel JNK cascade operating in LE cells during DC. Signaling via this JNK cascade has been shown to be required for the LE cell specific expression of the dpp gene.

We have attempted to understand the mechanism of how movements of the two large epithelial sheets of embryonic epidermis are orchestrated by the JNK and TGF-ß cascades, and the Rho subfamily of p21s during DC. Detailed phenotypic analysis of the distribution of the actin and myosin cytoskeleton, the putative focal complexes (Harden et al., 1996) and the putative Drac1/Dcdc42 effector molecule DPAK during DC, has revealed specific roles for Drac1, Dcdc42, JNK and TGF-ß signaling in the regulation of the cytoskeleton during DC. We show here that all mutants of genes within the JNK cascade share a common cytoskeletal phenotype, that is distinct from that shared by mutations in genes of the TGF-ß pathway. Genetic interactions between Drac1 and Djun (Hou et al., 1997), and Drac1 and DJNKK (hek, Glise and Noselli, 1997) and our analysis confirm that Drac1 can signal to an AP-1 complex (Dfos/Djun) in the nucleus, via the JNK cascade, to effect both assembly of cytoskeletal and other proteins, as well as initiate transcription of the dpp and puc genes. In contrast, mutations in TGF-ß pathway genes only moderately affect the levels of LE cytoskeleton, but cause an inappropriate movement of the epidermal cells, not seen in Drac1/JNK cascade mutants, and a loss of DPAK at the LE. In embryos mutant for TGF-ß pathway genes, LE cells are progressively pulled in towards the segment borders which causes a bunching of the epidermis. This phenotype is identical to that seen by blocking the endogenous Dcdc42 signal with a dominant-negative transgene (Harden et al., 1999). Our genetic analysis shows that only Dcdc42, and not Drac1, can signal downstream of the TGF-ß receptors during DC to regulate the dynamics of the DC process. We propose a model of how TGF-ß/Dcdc42 signaling can regulate the movements of the epidermis during DC using the putative effector molecule DPAK.

**MATERIALS AND METHODS**

**Fly strains**

Dominant negative (i.e. N17) and activated (i.e. V12) forms of Drac1 and Dcdc42 cDNAs cloned into the pUAST vector were a gift from L. Luo (Luo et al., 1994). UAS lines were expressed using the GAL4 system of Brand and Perrimon (1993). The GAL4 lines GAL4-2207, M-4 Hs-GAL4, and ptc-GAL4 were kindly provided by the Bloomington stock centre, J. Roote and E. Knust, respectively. Strains bearing the following alleles of mutations in genes that give a defective DC phenotype tkvD0F, puci35J, shnB15, pnrG, l(2)IA109, DjunIA109, kayb (Dfox) were originally isolated by Weihsa and Nusslein-Volhard (Nusslein-Volhard et al., 1984; Jurgens et al., 1984) and were obtained from the Bloomington Stock Centre, except for the strain Dflyfshp147E/Cyo (DNK/bsk) is described in Riesgo-Escovar et al., 1996) which was a gift from E. Hafen. The HS-Drac1N1704, HS-GAL4H4 and UAS-Ddc42DH7, HS-GAL42207 lines are described by Harden et al. et al., 1999). The tkv, HS-GAL42207/Cyo and tkv/B2Cyo;UASDdc42V12 lines were constructed for this work using standard Drosophila genetics. To facilitate scoring of homozygous mutant embryos during immunohistochemical analysis, all mutant chromosomes were placed over balancer chromosomes bearing a LacZ gene driven by either the frt or ubx promoter and homozygous mutant embryos identified by the absence of LacZ expression, visualised by anti-b-galactosidase (b-gal) antibody. The TAJ3 line bearing a constitutively activated version of the Tkv receptor under the control of the UAS promoter was a gift from M. O’Connor (Hoodless et al., 1996).

**GAL4 and heat shock expression of transgenes**

Unless otherwise stated, females from GAL4 lines were crossed to males from the UAS-Ddc42 and Drac1 transgenic lines and the progeny examined as embryos. When embryos were to be assessed for a cuticle phenotype, they were collected in two-hour periods at 25°C and aged at 25°C until 4 to 12 hours after egg laying (AEL).
They were then placed in vials and heat shocked in a waterbath set at 37°C. Following heat shock, embryos were aged at 21°C for at least 48 hours and subjected to cuticle preparation. When embryos were to be assessed by immunohistochemistry, they were collected at 25°C over a 4 hour period, aged at 25°C until 8 to 12 hours AEL, and heat shocked as above. Following heat shock, embryos were aged for 7 hours at 21°C and fixed for immunohistochemistry. In this study, all heat shocks were performed for 1 hour. Control embryos were collected from all crosses and were maintained at 25°C or 21°C prior to cuticle preparation or immunohistochemistry. For the rescue cross tkv/B2CyO; UASDcdc42V12 males were crossed to tkv, HS-GAL4207 CyO females which were left to lay at 21°C, as were the corresponding control collections. Embryos were raised at 21°C and then harvested and processed as for heat shock experiments.

Immunohistochemistry

Embryos to be stained were first dechorionated in 50% household bleach, washed in 0.01% Triton and fixed for 20-30 minutes in 1:1 4% paraformaldehyde in PBS (0.1 M NaCl, 10 mM phosphate buffer, pH 7.4): heptane. Devitellassion was performed by removing the aqueous phase containing fixative and washing the remaining heptane layer with an equal volume of either methanol, or 80% ethanol if embryos were to be phalloidin stained. Methanol/80% ethanol was exchanged for PBT (PBS with 0.1% Triton X-100) by washing for one hour with at least 3 changes of PBT. Prior to addition of antibodies embryos were blocked by washing in PBT containing 1% bovine serum albumin (BSA) for one hour. Incubations with primary antibodies were overnight at 4°C in PBT containing 1% BSA, serum albumin (BSA) for one hour. Incubations with primary antibodies were overnight at 4°C in PBT containing 1% BSA, serum albumin (BSA) for one hour. Incubations with primary antibodies were overnight at 4°C in PBT containing 1% BSA, subsequently embryos were washed at room temperature for one hour with at least 3 changes of PBT. Fluorescent detection of primary antibodies was done using either secondary antibodies directly labeled with Texas Red or FITC or with biotinylated secondary antibodies and streptavidin labeled with Texas Red or FITC (all materials from Vector Laboratories or Jackson Immunologicals, except anti-PY from U.B.I.). Secondary antibodies were diluted 1:200 in 1% BSA in PBT and incubated for at least two hours at room temperature. Stained embryos were washed in several changes of PBT for at least one hour, and if required, were incubated with a 1:100 dilution of labeled streptavidin in PBS for one hour. For F-actin staining, FITC-labeled or TRITC-labeled phalloidin (Sigma) was added to a final concentration of 1 µg/ml and incubated for the last half an hour of the streptavidin staining. Embryos were washed in several changes of PBS and mounted in Vectashield (Vector Laboratories). Fluorescent stainings were viewed on either a Bio-Rad MRC 600 or 1024 confocal laser scanning microscope. As heat-shock and the ectopic expression of the various transgenes retard as well as having dramatic effects on development, it is very difficult to extrapolate the age of the embryos in terms of ‘hours after egg laying’. We have based our estimations of early, middle and late in DC on the relative degrees of: ventral nerve-cord development, the presence or absence of PNS organs and where possible, the amount of dorsal ward epidermal migration and head involution.

Cuticle preparations

Cuticles were prepared as previously described (Harden et al., 1999).

RESULTS

Signaling by the Drac1/JNK cascade is required for the recruitment of cytoskeletal components to the LE during DC

Various markers allow one to evaluate the integrity of the LE during DC. These include F-actin, myosin (Young et al., 1993) and the triangular nodes phosphotyrosine (PY) present at the LE in putative focal complexes (Harden et al., 1996). The presence or absence of DPAK, a putative downstream effector for Drac1 and Dcdc42, which accumulates along the LE reaching particularly high levels in the LE cells flanking the segment borders (Harden et al., 1996), can also be evaluated. We therefore examined the levels of F-actin, myosin, PY and DPAK in embryos homozygous for mutations in the genes that have been shown to be members of the JNK cascade: bsk (JNK) (Fig. 1E-H), h2IA109 (Djun) (Fig. 1I-L) and kay (Dfos) (Fig. 1M-P), and compared them to the levels found in wild-type embryos (Fig. 1A-D) and embryos in which dominant negative transgenes of Rho subfamily members had been induced (Drac1N17, Fig. 1Q-T; Dcdc42N17, Fig. 2Q-T). All JNK cascade mutants displayed a common cytoskeletal phenotype. These failed to show complete elongation of the dorsal epidermis (Fig. 1E,I,M; Fig. 3I) after germband retraction and, in all cases, the accumulation of actin and myosin normally seen at the LE (Fig. 1A,B) were absent (Fig. 1E,F,I,J,M,N). We also saw a concomitant loss of PY nodes (Fig. 1G,K,O) and DPAK staining (Fig. 1H,L,P) from the LE. We found that the JNK-pathway phenotypes closely resembled those produced by expression of Drac1N17 (Fig. 1Q-T) (Harden et al., 1995, 1996, 1999) suggesting that a Drac1-mediated JNK cascade is required for the assembly of leading edge architecture in these cells.

Mutations in the genes of the TGF-β pathway and expression of Dcdc42N17 cause a common DC phenotype that is distinct from that produced by JNK pathway mutants and expression of Drac1N17

Mutations in tkv and put, as well as other components of the TGF-β pathway in flies, are defective in DC. We wanted to determine if there was any phenotypic overlap between mutations in genes of the JNK-pathway, which produce the Dpp signal, and the TGF-β pathway, the proposed target for secreted Dpp protein. As before, we examined the levels of actin, myosin, PY, and DPAK in embryos homozygous for mutations in the genes that have been shown to participate in the TGF-β/Dpp signaling pathway during DC: shn (Fig. 2I-L), pnr (Fig. 2M-P), tkv (Fig. 2A-D) and put (Fig. 2D-G). Early in closure all of these mutants maintained appreciable accumulations of actin, myosin and PY nodes at the LE (Fig. 2A-C, E-G, I-K, M-O), in contrast to what is seen with mutants in JNK-cascade signaling and following Drac1N17 expression. We also looked at the overall morphology of the epidermis early and late in DC using anti-phosphotyrosine staining of JNK/TGF-β pathway mutants (Fig. 3). In embryos mutant for tkv, or the other TGF-β pathway genes (data not shown), epidermal cells initially appear to elongate correctly (Fig. 3B,C) but instead of continuing dorsal-ward their migration becomes misdirected in an anterior-posterior direction (Fig. 3D). Regions of the LE are seen in which cells of several segments are drawn together into points of focus, pulling the intervening segments apart. This produces a phenotype of epidermal bunching late in DC that is common to all TGF-β mutants (Fig. 3E-H). We clearly see the initial elongation of the more lateral epidermal cells, below the first row, in tkv mutants (Fig. 3C). Their elongation is lost later in DC, presumably due to the formation of epidermal bunches. After the segments are pulled into bunches it is difficult to distinguish which are the LE cells as elevated actin, myosin and PY are only seen at the focal points of the constrictions (data not
shown). Mutations in tkv, put, pnr and shn (Fig. 2D,H,L,P) all abolish the accumulations of DPAK that are normally present in the LE cells and at the segment borders (Fig. 1D) (Harden et al., 1996), even prior to the formation of these bunches. Induction of a Dcdc42N17 transgene also causes loss of DPAK protein from the LE and segment borders, and the epidermal bunching phenotype (Fig. 2Q-T) (Harden et al., 1999). Bunching of the epidermis was not seen with Drac1N17 expression, or in JNK pathway mutants (kay, Dfos mutant Fig. 3J). Therefore, members of the TGF-β pathway and Dcdc42 appear to have common roles in the control of epidermal morphology, during DC, distinct from those of Drac1 and the JNK pathway. Dcdc42 and the TGF-β pathway appear to direct migration of the epidermis and elevate DPAK levels at the LE and in the segment border cells, and may thus act in a common pathway to control the mechanics of the closure process.

Co-expression of an activated tkv transgene can partially suppress the effects of Drac1N17 but not the effects of Dcdc42N17

Drac1 has been shown to signal upstream of the JNK cascade (Hou et al., 1997; Glise and Noselli, 1997). It has been proposed that Dcdc42 also has the capacity to signal in the JNK cascade during DC (Glise and Noselli, 1997). However, our analysis of cytoskeletal phenotypes suggests that Dcdc42 may play a greater role in TGF-β signaling than in the JNK cascade. To examine the hierarchical relationship of the Drac1 and Dcdc42 genes with respect to TGF-β receptor signaling, we co-expressed a constitutively activated tkv transgene (TAJ3, Hoodless et al., 1996) along with either HS-Drac1N17 or UAS-Dcdc42N17 transgenes using HS-GAL4. We found that expressing TAJ3 partially rescued the DC defect induced by Drac1N17 at the level of the leading edge cytoskeleton, as there was some replacement of F-actin (Fig. 4A,B) and other cytoskeletal components, (data not shown) at the LE that is normally removed by expression of Drac1N17 (Harden et al., 1999). This result is not surprising as some mutations of JNK-cascade genes can also be partially suppressed by expression of a dpp transgene. In contrast, expression of TAJ3 appeared to have no effect on the Dcdc42N17-induced phenotype. There was no change either at the level of the cuticle (data not shown), or at the level of the cytoskeleton. Embryos co-expressing TAJ3 and UAS-Dcdc42N17 transgenes still displayed the characteristic bunching of segments (Fig. 4C,D) seen in embryos expressing UAS-Dcdc42N17 alone (Fig. 3I). These results suggest that tkv...
can act downstream of Drac1, and that Dcdc42 may lie downstream of tkv.

**Rescue of the tkv mutant phenotype by expression of an activated Dcdc42 transgene**

In contrast to Drac1, defects in Dcdc42 signaling cannot be bypassed by ectopic activation of the TGF-β pathway with activated Tkv, indicating that Dcdc42 may be downstream of tkv during DC. To determine if this epistasis is due to Dcdc42 acting downstream of the TGF-β receptors, and not in a parallel pathway in DC, we examined if expression of a constitutively activated version of Dcdc42 (UAS-Dcdc42V12) could rescue the tkv phenotype, by expressing UAS-Dcdc42V12 in embryos homozygous for the tkv mutation using the HS-GAL4^{2207} driver. Expression of Dcdc42V12 was able to rescue the DC phenotype of the tkv mutation. The most efficient rescue of the tkv defect, without inducing any of the defects characteristic of higher level inductions of UAS-Dcdc42V12 (Harden et al., 1999), was obtained by raising embryos from crosses of females of the genotype: tkv, HS-GAL4^{2207}/CyO to males of the genotype: tkv/Blue2CyO;UAS-Dcdc42V12 (hereafter referred to as ‘rescue cross’) at constant temperatures, and not by any regimen of heat-shocks. Presumably, this method allowed enough stable expression of GAL4, and thus Dcdc42, from the heat shock promoter to overcome the defect in tkv signaling (see Fig. 6). At 21°C we observed the most dramatic lessening of the frequency of the DC defect; the large dorsal hole found in homozygous tkv embryos (Fig. 5B,C) decreases from 27.7%, close to the predicted one quarter, in the control strain tkv;HS-GAL4^{2207}/CyO, down to 6.2% in the rescue cross. Expression of Dcdc42V12 under these conditions allowed most of the embryos with large dorsal holes (Fig. 5B,C) to progress to a much more complete form of closure resulting in less severe phenotypes, generally a puckering or scarring of the dorsal surface (Fig. 5D). It is important to note that these conditions did not cause significant levels of Dcdc42V12-specific cuticular phenotypes (Harden et al., 1999) such as defects in cuticle formation or germ band retraction defects. The dramatic reduction in the frequency of the large open holes (from 27.7% to 6.2%, n>1000) suggest that Dcdc42 has the capacity to act downstream of the tkv receptor in the control of DC. The results of the rescue cross are represented graphically in Fig. 6.

To determine the nature of tkv suppression by Dcdc42V12 we examined, by PY staining, the cellular morphology of embryos from the rescue cross raised at 21°C. Embryos resulting from the rescue cross (Fig. 4E,F) rarely showed any of the misdirected movement and segmental bunching observed in TGF-β mutants or following expression of Dcdc42N17 (Fig. 3) and completed DC (Fig. 4G,H). This near complete suppression of the bunching of segments in tkv

![Fig. 2. Effects on the LE cytoskeleton of mutations in various TGF-β pathway genes and comparison with embryonic cytoskeletal phenotypes induced by expression of a Dcdc42N17 transgene. Confocal fluorescence micrographs show embryos in the early stages of DC stained with; phalloidin to detect F-actin (A,E,I,M,Q), anti-nonmuscle myosin (B,F,J,N,R), anti-PY (C,G,K,O,S), and anti-DPAK antibodies (D,H,L,P,T). Staining of tkv (A-D), put (E-H), pnr (I-L), shn (M-P) homozygous, and HS-GAL4^{2207};UAS-Dcdc42N17 (Q,R) and ptc-GAL4;UAS-Dcdc42N17 (S,T) embryos show near normal levels of LE F-actin (A,E,I,M,Q) and myosin (B,F,J,N,R), normal triangular nodes of PY (C,G,K,O,S), but a loss of DPAK (D,H,L,P,T; lower magnification see Fig. 1) from the LE and segment border cells.](image-url)
mutants by ectopic expression of UAS-Dcdc42V12 suggests that activating Dcdc42 can bypass the need for Tkv signaling during DC. These results indicate that Dcdc42 lies downstream of the TGF-β receptors, and not in a parallel pathway, in control of the migration of the epidermis during DC. DPAK is a putative effector for Dcdc42, and its accumulation at the LE and segment borders depends on Dcdc42/TGF-β signaling. As such, it is tempting to speculate that it too participates in the control of epidermal migration during DC. We therefore checked to see if there was any rescue of the levels and localisation of DPAK protein to both the LE and the cells adjacent to the segment borders (Fig. 4I,J).

**DISCUSSION**

**JNK and TGF-β signaling pathways play distinct roles during DC**

Embryos homozygous for mutations in JNK cascade genes exhibit little or no elongation of the lateral epidermis and lack components of the cytoskeleton at the LE. In addition, the products of the dpp and puc genes, are lost from the LE at the onset of DC. By comparison, mutations in the TGF-β pathway genes have milder effects on the LE cytoskeleton, which is relatively intact, as is the initial expression of Dpp protein (M. G. Ricos, unpublished data). The earliest visible defect in the TGF-β class of mutants is the loss of the DPAK protein from the LE and in the segment border cells. There is some elongation of the lateral epidermis, not seen in JNK pathway
mutants, but this becomes misdirected and the closing epidermis gets pulled into a series of foci instead of migrating dorsally in an orderly fashion. Our results clearly indicate that the role of the JNK cascade cannot solely be initiation of dpp expression as mutations in all identified TGF-β pathway genes have phenotypes distinct from those of mutations in the JNK cascade genes. JNK cascade signaling is required for both the complete assembly of the LE cytoskeleton and production of Dpp, whereas the TGF-β pathway mainly participates in the regulation of the migration process, and only strongly affects the LE levels of DPAK, and not other LE components.

The *Drosophila melanogaster* homologue of p65PAK, DPAK (Harden et al., 1996), appears to be regulated by both Drac1 and Dcdc42 during DC. We show a decrease in Drac1/JNK signaling can remove DPAK expression from the LE cells. However, it is not possible to determine if this absence of DPAK protein from the LE is due to a direct effect on DPAK levels, such as a specific requirement for JNK signaling via the AP-1 complex for the induction of DPAK transcription. More likely, loss of DPAK is a secondary effect from either the loss of the cytoskeletal scaffold that is required for DPAK binding to the LE in these cells, or due to the loss of expression of the Dpp ligand required for activating the TGF-β pathway.

Our analysis of the TGF-β mutant phenotypes differs from that previously reported for *tkv* (Riesgo-Escovar and Hafen, 1997b) and the conclusions we draw also differ significantly. It was previously postulated that signaling via the TGF-β pathway is required for elongation of the cells below the LE. However, we observe normal elongation of all of the epidermal cells in TGF-β pathway mutants in the initial stages of DC. The bunching of the LE later in DC appears to cause a relaxation of the more ventrally located epidermal cells, giving the impression

![Fig. 4. Genetic interactions of Drac1 and Dcdc42 with tkv. (A) Phalloidin stained TAJ3;HS-Drac1N17104,HS-GAL4 embryo heat shocked during DC showing some elongation of the LE. (B) High magnification view of A showing presence of F-actin at the LE. (C) Anti-PY stained TAJ3;Dcdc42N17:HS-GAL4 embryo still showing Dcdc42N17-induced bunching of segments despite the expression of activated Thickveins. (D) High magnification view of C highlighting the bunching phenotype. (E) Anti-PY stained tkv,HS-GAL4,tkv;UASDcdc42V12/+ embryo raised at 21°C during DC showing suppression of the bunching normally seen in tkv/tkv embryos. (F) High magnification view of E showing no bunching at the LE and relatively normal PY nodes along the LE. (G) Anti-PY stained tkv,HS-GAL4,tkv;UASDcdc42V12/+ embryo raised at 21°C during late stage of DC showing a near complete DC and minor puckering of the dorsal epidermis. (H) High magnification view of G. (I) Anti-DPAK stained tkv,HS-GAL4,tkv;UASDcdc42V12/+ embryo raised at 21°C during DC showing replacement of DPAK protein at the LE and in the cells adjacent to the segment borders. (J) High magnification view of I.](image-url)
that only the LE cells have elongated. This may explain the earlier interpretation of the TGF-β mutant phenotype.

Results from mammalian cell culture studies have shown that Rac and Cdc42 can signal to the nucleus via JNK/SAPK signaling cascades (Minden et al., 1995; Coso et al., 1995; Olson et al., 1995) and there is evidence that this may be mediated by PAK activity (Brown et al., 1996; Zhang et al., 1995). However this is not always the case as the ability of PAK to link p21 signaling to a JNK/SAPK pathway depends on the context in which it is assayed (Lamarche et al., 1996). The nuclear component of Drac1/JNK signaling is required for expression of both Dpp and the phosphatase Puc. However it is not known if the induction of dpp and puc expression is directly mediated by binding of the active AP-1 complex (Djun/Dfos) to the dpp and puc promoters. Expression of a dominant negative Drac1 transgene causes a phenotype identical to those exhibited by mutations in genes of the JNK signaling cascade, which is characterised by a failure to accumulate actin and myosin along the LE, a distinct absence of LE focal complexes, as demonstrated by the lack of PY nodes, and the absence of DPAK at the LE. Previous observations that co-expression of an activated Djun transgene can partially suppress the effects of Drac1N17 transgene expression (Hou et al., 1997), and the ability of hep (JNKK) mutants to block the effects of a Drac1V12 transgene with respect to induction of dpp and puc expression (Glise and Noselli, 1997), clearly demonstrate the participation of Drac1 in a JNK signaling pathway during DC. That blocking signaling via this cascade can prevent both the expression of specific genes and assembly of the cytoskeleton in the first row of epidermal cells suggests that the pathway may be required for specification of LE cell identity. To date there are no mutations or transgenes that can selectively remove either the cytoskeleton or gene expression from the LE. As such it is not clear if the processes of specification, induction of gene expression, and recruitment and organisation of cytoskeletal components are separable.

In cell culture, microinjection of activated Rac or Cdc42 proteins can rapidly induce cytoskeletal reorganisation, and cell shape changes in under 2 minutes (Ridley and Hall, 1992; Ridley et al., 1992; Kozma et al., 1995; Nobes and Hall, 1995).
There is no requirement for JNK-mediated nuclear signaling (Joneson et al., 1996; Lamarche et al., 1996), or new RNA and protein synthesis (Thomas Leung and Xiang-Qun Chen, personal communication) to effect these changes in the cytoskeleton. In cell culture, rearrangement of the existing pool of cellular proteins (i.e. actin, myosin and DPAK) is apparently sufficient to effect the observed cell shape changes, however, the demands on individual cell shape change during tissue morphogenesis in vivo are likely to be far greater. The requirement for both cytoplasmic and nuclear components of both Drac1/JNK and TGF-β/Dcdc42 signaling in vivo during DC is probably not due to any novel mechanism for Rho subfamily signaling in flies, but most likely reflects the requirement for increased levels of proteins, cytoskeletal or otherwise, to effect cell shape changes during morphogenesis. Signals transmitted by the Rho subfamily in vivo may require a nuclear component, to effect increased production of proteins, and a cytoplasmic component to effect rearrangement of the cytoskeleton.

**Dcdc42 in the Dpp/Tkv pathway**

We have previously shown that expression of dominant negative Dcdc42 can interrupt the closure process in a distinct way from Drac1 (Harden et al., 1999). Here we show that Dcdc42 acts downstream of the TGF-β receptors. Blocking the activity of Dcdc42 is phenotypically equivalent to blocking signaling in the TGF-β pathway via mutations in either the receptors tkv and put, or the downstream transcriptional regulators pnr and shn, and does not completely remove the LE cytoskeleton or prevent epidermal migration per se, but causes misregulated movement of the LE cells. This inappropriate movement of LE cells causes the majority of epidermal segments to be pulled together into several focal points and the intervening segments to be torn apart. Bunching of the leading edge may result for a number of reasons. Hyper-contraction of the postulated acto-myosin contractile apparatus could result in the production of an excessive antero-posteriorly directed force which could overconstrict the LE cells causing them to be drawn towards each other. If this force was strong enough it could break the contacts between the LE cells and the underlying tissue, and each other, and cause the shearing of some segments that we observe. Dcdc42 may thus be playing a role in regulating the strength of the contraction along the leading edge presumably by regulating the activity of myosin. This, however, is unlikely as there are no reports of Cdc42 affecting myosin activity and it would be difficult to explain the equivalent action of the TGF-β pathway in all of the leading edge cells given the fact that tkv expression is not equally present in all of the LE cells (Affolter et al., 1995). Dpp protein is also not evenly distributed throughout the epidermis. During DC, it is concentrated at the LE with the greatest levels adjacent to the segment borders (M. G. Ricos, unpublished data). An alternative explanation may be that there are different levels of TGF-β signaling across the embryonic epidermis that normally regulate the dynamics of cell movement to achieve an orderly epidermal migration, possibly through regulating DPAK.

PAK can contribute to the dissolution of actin structures and focal complexes in mammalian cells (Manser et al., 1997). We have observed that, during DC, expression of an activated form of Dcdc42 can cause both an elevation of DPAK at the LE, as well as a loss the entire LE cytoskeleton at a lower frequency (Harden et al., 1999). We postulated that this excessive upregulation, and probable activation, of DPAK by Dcdc42 can lead to the subsequent dissolution of all of the leading edge cytoskeletal components (Harden et al., 1999). Previously we reported transient breaks in the integrity of the LE cytoskeleton occurring in cells flanking the segment borders which are particularly enriched in DPAK, and proposed DPAK as the mediator of these transient breaks (Harden et al., 1996). It is interesting to note that the cells flanking the segment borders have the highest LE levels of tkv transcripts and are adjacent to the highest concentrations of Dpp protein and that Dcdc42V12 expression, which can rescue the tkv phenotype, can also replace the lost DPAK protein at the LE and near the segment borders in tkv embryos. We have previously demonstrated a role for DrhoA in the maintenance of the LE cytoskeleton in the cells flanking the segment borders (Harden et al., 1999). As antagonism between RhoA and Cdc42 signaling has been demonstrated in some mammalian cell types (Lim et al., 1996; Kozma et al., 1997; Allen et al., 1997), it is tempting to speculate that, during DC, signaling from DrhoA and Dcdc42 may compete to regulate the LE cytoskeleton at the segment borders.

**A new model for signaling to the cytoskeleton**

A consideration of our data and the work of others leads us to postulate a novel mechanism for regulation of the cytoskeleton in DC by the Drac1/JNK and TGF-β/Dcdc42 pathways. During DC, Drac1-mediated activation of a JNK cascade induces the production of both the Puc phosphatase, a negative regulator of...
insights into how different signaling pathways can interact to DPAK in Dcdc42-mediated TGF-β signaling pathways in DC, in particular the involvement of mechanics of epidermal migration. Further study of these path-ways do it.

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JNK signaling (Martin-Blanco et al., 1997), and Dpp, and directs assembly of the LE cytoskeleton. Drac1 is also responsible for controlling the normal cell shape changes of the amnioblasts during DC (N. Harden and M. G. Ricos, unpublished observations). Thus, Drac1 and the JNK cascade have major roles in initiating cell shape changes during DC. Dpp produced by the JNK cascade is postulated to activate the TGF-β/β pathway via type-I and type-II TGF-β receptors Punt and Tkv leading to activation of Dcdd42, and a series of transcription factors shn, pnr, and possibly Mad. Dpp signaling, during DC, is not a simple extension of the Drac1/JNK pathway as its downstream components do not assemble the LE architecture, but acts to control the mechanics of the DC process to bring about orderly movement of the embryonic epidermis over the amnioblasts. We therefore propose the following model for the mechanism of control of DC by the Drac1/JNK and Dcdd42/TGF-β pathways (Fig. 7): Drac1/JNK signaling initiated by an as yet unknown factor assembles cytoskeletal components (F-actin, myosin and focal complexes) and other proteins (Dpp, Puc, and DPAK) in the LE cells and initiates cellular migration. Dpp-activated signaling controls the dynamics of epidermal migration, via Dcdd42 and the TGF-β pathway, through the TβR kinase DPAK which transiently down regulates the LE cytoskeleton at the segment borders (Fig. 7). Transient downregulation of the actin cytoskeleton and focal contacts near the segment border cells is likely to cause local relaxation of the anterior-posterior tension along the LE. Such transient relief of tension may then limit excessive migration of LE cells towards each other and prevents the bunching and shearing of epidermal segments that occurs following impairment of TGF-β/Dcdd42 signaling. Cells flanking segment borders are potential regions of high TGF-β signaling as they are adjacent to the highest local concentrations of Dpp protein and they have high levels of DPAK protein and transcripts for the Tkv receptor. Cells flanking segment borders are the only places where transient downregulation of the LE cytoskeleton is ever seen in wild-type embryos during DC. As such we propose that the role of Dcdd42/TGF-β signaling is the induction of DPAK to down regulate the LE cytoskeleton at the segment borders, introducing a degree of flexibility of the LE during the DC process (Fig. 7).

Our study has demonstrated distinct roles for the Drac1-mediated JNK pathway and the TGF-β pathway in control of the cytoskeleton during DC. We have also shown that only Dcdd42 and not Drac1 can participate in signaling downstream of the TGF-β receptor Tkv, during DC, to regulate the mechanics of epidermal migration. Further study of these signaling pathways in DC, in particular the involvement of DPAK in Dcdd42-mediated TGF-β signaling, should provide insights into how different signaling pathways can interact to orchestrate epidermal morphogenesis.

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