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Dcdc42 acts in TGF-β signaling during *Drosophila* morphogenesis: distinct roles for the Drac1/JNK and Dcdc42/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 *Dcdc42*, 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 for 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, Dcdc42, 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, Dcdc42 are involved in the initial assembly and/or maintenance of the LE cytoskeleton. Dcdc42 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-β) 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-β 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-β 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-β receptors and have been characterised in many biological systems (reviewed by Derynck and Zang, 1996; Wrana and Attisano, 1996; Massague et al., 1997). Intracellular MAPK/JNK-cascade-like kinases (Yamaguchi et al., 1995; Moriguchi et al., 1996; Atfi et al., 1997a; Wang et al., 1997) and some of the small GTPases (Atfi et al., 1997b; Mucsi et al., 1996) can also effect signaling downstream of TGF-β receptors.

Involvement of a TGF-β signaling pathway operating during DC, 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-β 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-β 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-β family member, Decapentaplegic (Dpp), produced in the LE cells has been postulated to bind to, and initiate TGF-β 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-β 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-β 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-β pathway. Genetic interactions between *Drac1* and *Djun* (Hou et al., 1997), and *Drac1* and *DJNKK* (*hep*, 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-β 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-β 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-β receptors during DC to regulate the dynamics of the DC process. We propose a model of how TGF-β/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 tkv^{strID}, put^{135}, shn^{1B}, pnr^{7G}, *l(2)IA109* (*Djun* ^{IA109}), kay^{7p} (*Dfos*) were originally isolated by Weischaus and Nusslein-Volhard (Nusslein-Volhard et al., 1984; Jurgens et al., 1984) and were obtained from the Bloomington Stock Centre, except for the strain *Df(2L)flp147E/CyO* (*DJNK/bsk* is described in Riesgo-Escovar et al., 1996) which was a gift from E. Hafen. The *HS-Drac1N17104,HS-GAL4*^{M4} and *UAS-Dcdc42N17,HS-GAL4*^{2207} lines are described by Harden et al. (1999). The *tkv,HS-GAL4*^{2207}*/CyO* and *tkv/B2CyO;UASDcdc42V12* 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 *ftz* or *ubx* promoter and homozygous mutant embryos identified by the absence of *LacZ* expression, visualised by anti-β-galactosidase (β-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-*Dcdcd42* 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-GAL4^{2207}/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. Devitellinisation 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, subsequently embryos were washed at room temperature for one hour with at least three 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 1:1000 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), *l(2)IA109* (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. 3J) 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

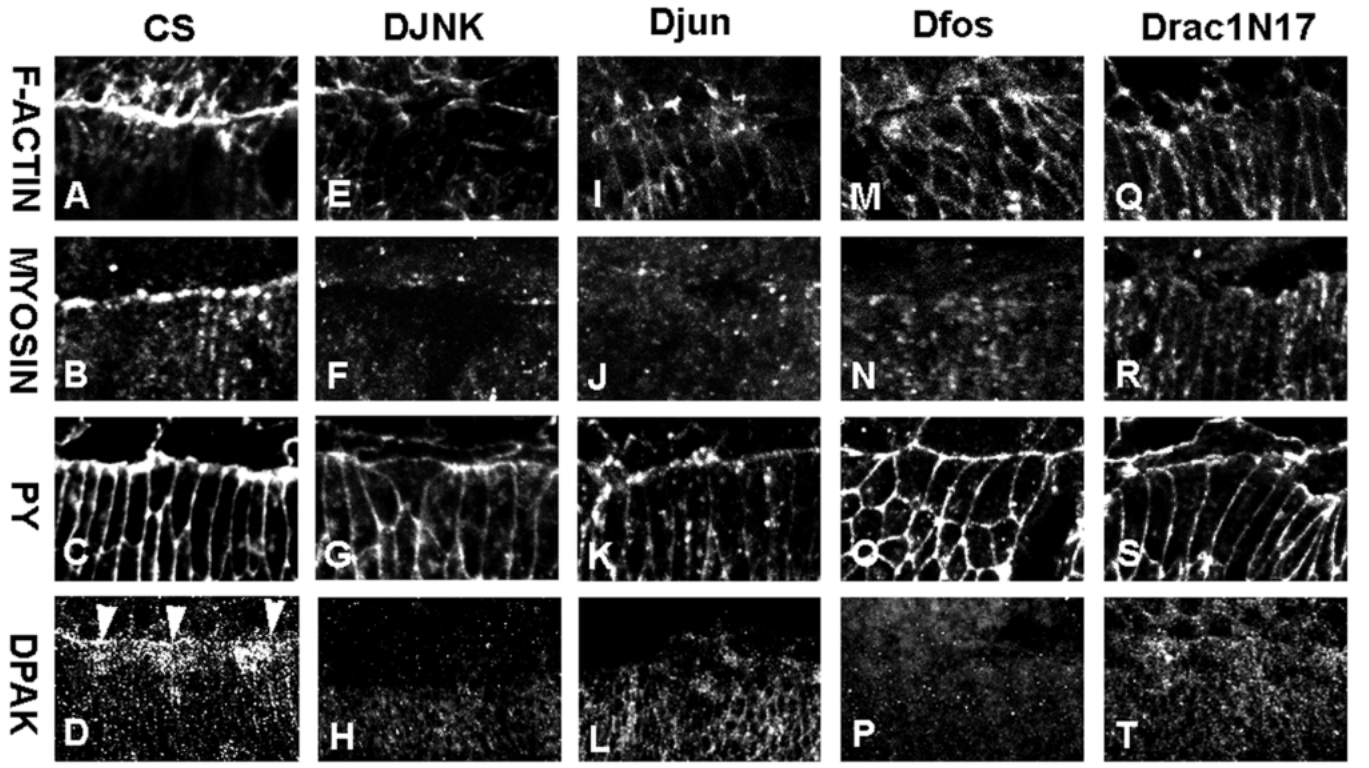

Fig. 1. Comparisons of the leading edge cytoskeleton of mutant embryos of various JNK cascade genes with Drac1N17-expressing embryos and WT embryos. Confocal fluorescence micrographs show the boundary between the amnioserosa (top of each micrograph) and the epidermis during early stages of DC. Anterior is to the left and dorsal is up in this and other figures unless otherwise stated. Embryos were stained with phalloidin to detect F-actin (A,E,I,M,Q), anti-nonmuscle myosin antibodies (Kiehart and Feghali, 1986) (B,F,J,N,R), anti-phosphotyrosine antibodies (C,G,K,O,S), or (A-D) control staining of CS embryos showing F-actin accumulation (A), elevated and punctate distribution of myosin (B), triangular nodes of PY (C) at the LE and elevated DPAK (D) at the LE and in segment border cells (arrowheads). Stainings of *Df(2l) flp147E* (E-H), *l(2) IA109* (I-L), *kay* (M-P) homozygous and *HS-GAL4^{M-4};UAS-Drac1N17.1* (Q-T) embryos show loss of F-actin (E,I,M,Q), myosin (F,J,N,R), PY nodes (G,K,O,S) and DPAK (H,L,P,T) from the LE of the epidermis during DC. Panels showing embryos stained with anti-DPAK antibodies (D,H,L,P,T) are presented at a lower magnification to show several segments. Drac N17 expression was induced as close as possible to the start of DC, but late enough so as to avoid the complications of germband retraction defects and gross disruption of the epidermis that can arise with earlier induction (Harden et al., 1995, 1999).

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-Drac1N17104* 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 germband 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*

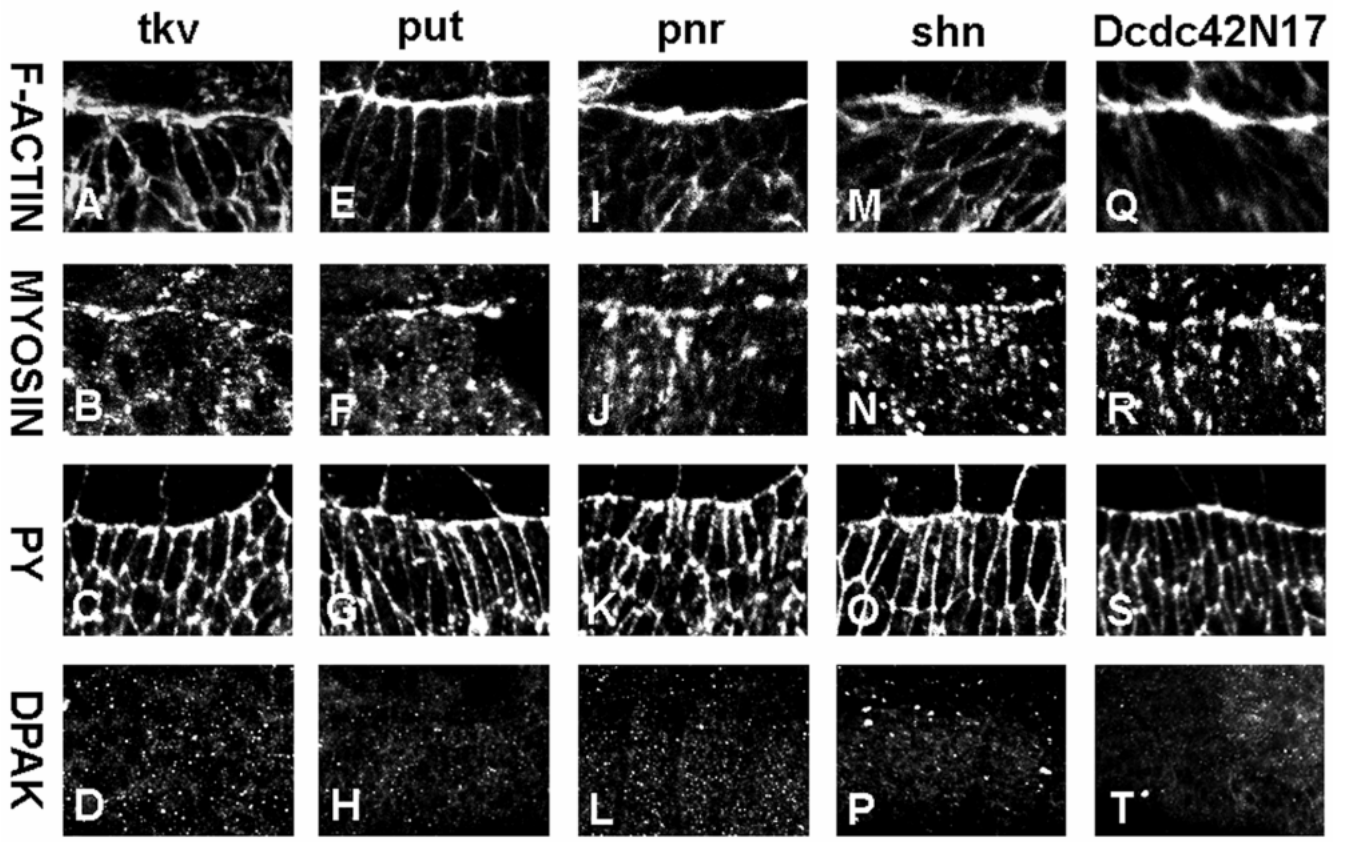

Fig. 2. Effects on the LE cytoskeleton of mutations in various TGF-β pathway genes and comparison with embryonic cytoskeletal phenotypes induced by expression of a Dcdc42N17 transgene. Confocal fluorescence micrographs show embryos in the early stages of DC stained with; phalloidin to detect F-actin (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.

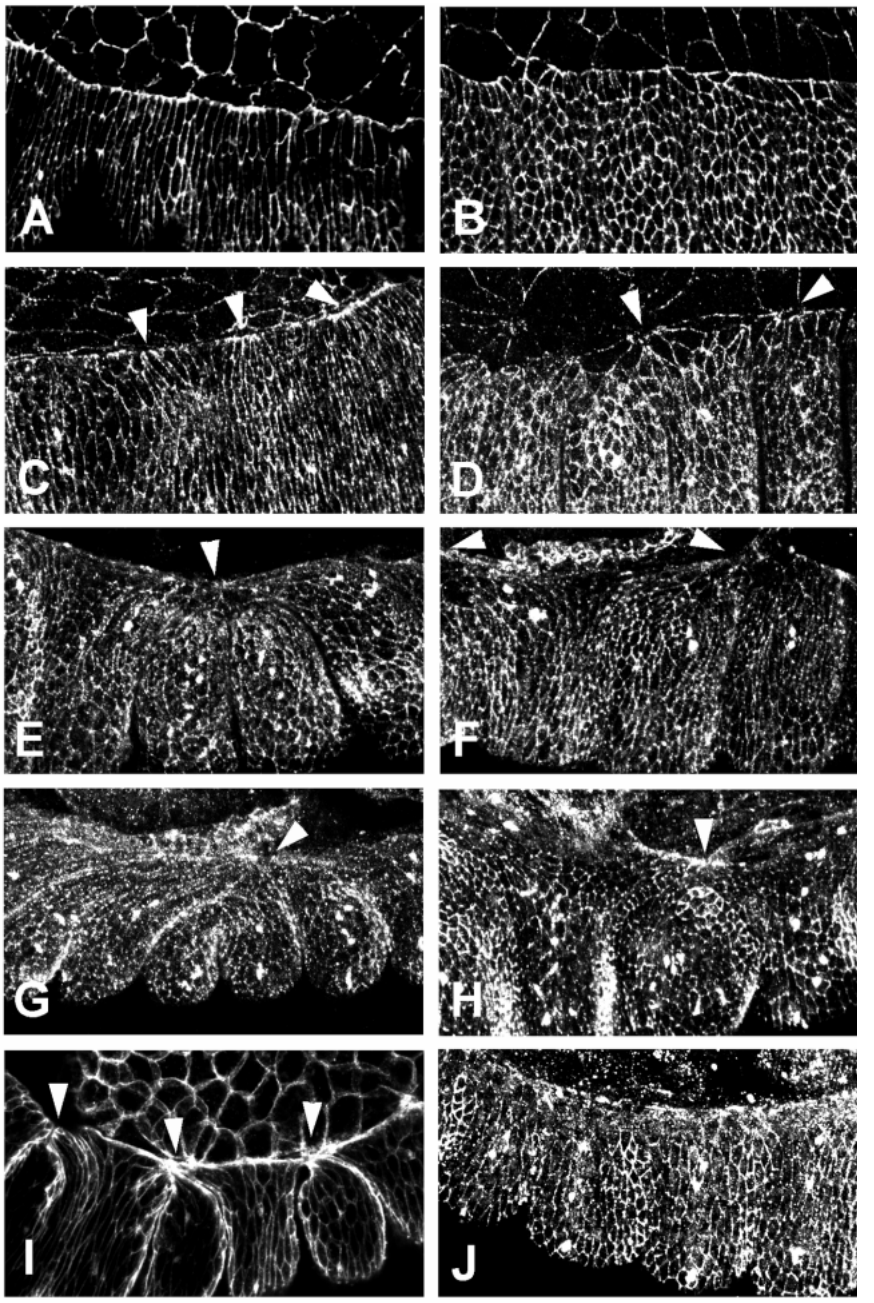

Fig. 3. Cell shape in TGF-β pathway mutant embryos and embryos expressing Dcdc42N17 during the course of DC. Confocal fluorescence micrographs of embryos stained with anti-PY antibody to reveal cell shapes at various times during DC. (A) CS embryo showing regular elongation of the epidermal cells. (B-E) *tkv* homozygous mutant embryos during DC. (B) Initial elongation of the first row of epidermal cells is similar to that seen in WT embryos (Young et al., 1993). (C) Subsequent elongation of epidermal cells below the dorsal most row of cells in *tkv* mutant embryos is similar to that of WT embryos (A) except for the slight clustering of some PY nodes along the LE (arrowheads), which may reflect the onset of epidermal bunching. (D) Embryos showing the beginnings of pulling of the leading edge into points of focus (arrowheads), and some lessening of the contact of the LE with the amnioserosa cells. At this stage there is already some reduction in the degree of elongation of the epidermal cells along the dorsal-ventral axis. (E) *tkv/tkv* embryo at the end of DC showing pronounced bunching of segments (arrowheads) characteristic of the TGF-β pathway. (F) *put/put* embryo, at the end of DC, showing bunching of epidermal segments (arrowheads). (G) *pnr/pnr* embryos at the end of DC showing similar bunching of epidermal segments (arrowheads). (H) Staining of late DC *shn/shn* embryos also displaying bunching of epidermal segments (arrowheads). (I) Staining of heat-shocked *HS-GAL4^{2207};UAS-Dcdc42N17* embryos at a late stage of DC reveals a bunching of epidermal segments (arrowheads). (J) *kay/kay* embryo at the end of DC showing lack of elongation of the LE cells and no bunching of the epidermis.

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 in embryos from the rescue cross at 21°C. We were able to observe a reasonable replacement of the accumulations 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

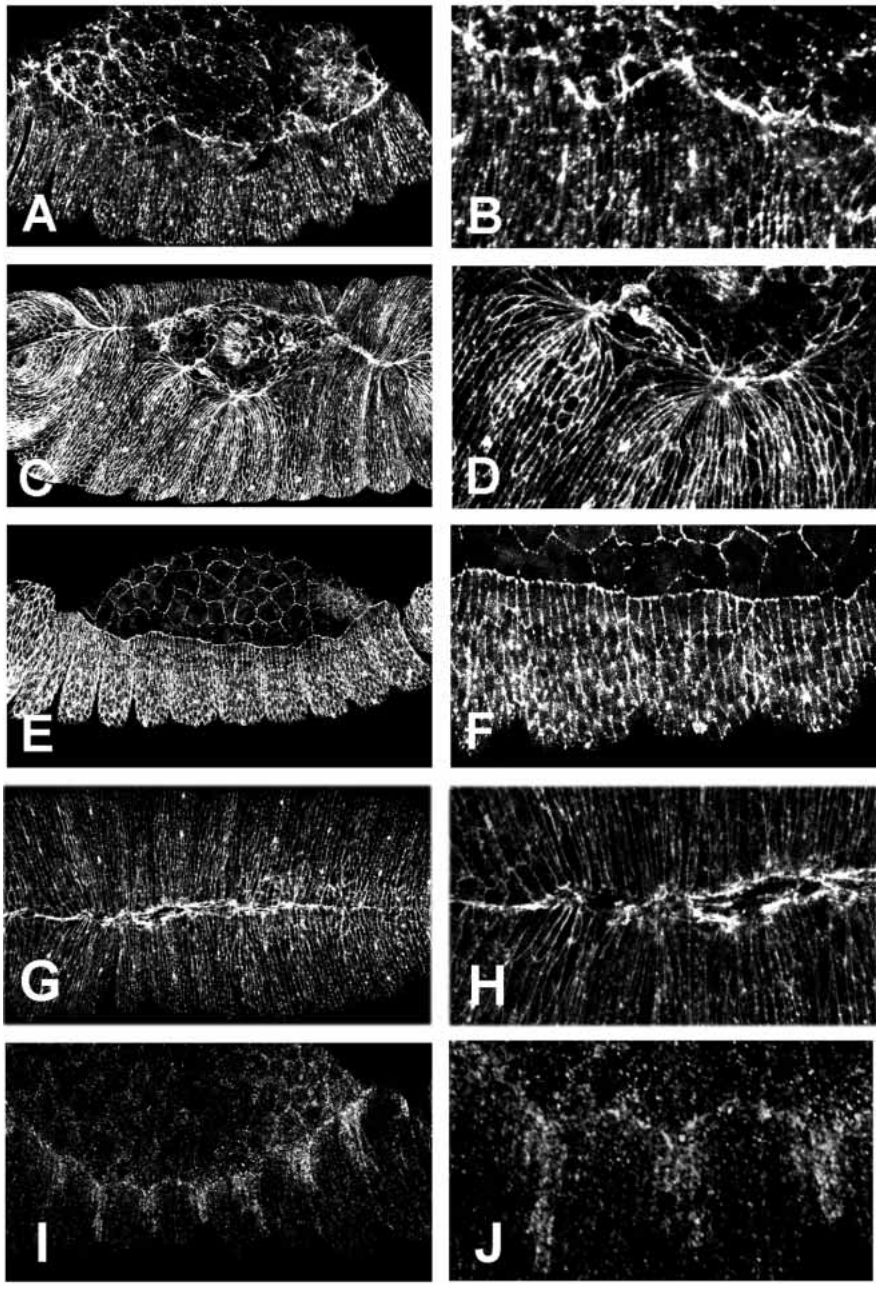

Fig. 4. Genetic interactions of Drac1 and Dcdc42 with *tkv*. (A) Phalloidin stained *TAJ3;HS-Drac1N17104,HS-GAL4* ^{M4} embryo heat shocked during DC showing some elongation of the LE. (B) High magnification view of A showing presence of F-actin at the LE. (C) Anti-PY stained *TAJ3;Dcdc42N17:HS-GAL4*^{2207} 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*^{2207}*/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*^{2207}*/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*^{2207}*/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.

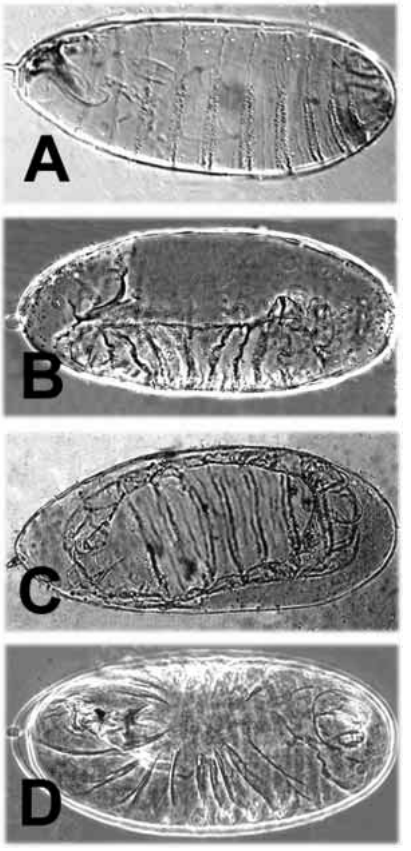

Fig. 5. Rescue of *tkv* by Dcdc42V12: cuticle preparations. Comparison of cuticle preparations of CS (A), *tkv/tkv* (B,C) and *tkv:HS-GAL4^{2207}/tkv;UASDcdc42V12/+* (D) embryos; in all cases dorsal is up and anterior is to the left, except C and D which are dorsal views. (A) Wild-type cuticle pattern showing normal pattern of denticle belts on ventral surface and complete dorsal cuticle indicative of complete closure of the dorsal epidermis. (B) *tkv/tkv* embryos displaying characteristic dorsal open phenotype, only the ventral cuticle and denticle belts are visible demonstrating a complete failure of the dorsal epidermis to close to any degree. (C) Dorsal view of *tkv/tkv* embryo showing characteristic large dorsal hole. (D) Dorsal view of *tkv,HS-GAL4^{2207}/tkv;UASDcdc42V12/+* embryo grown at 21°C showing a high degree of rescue of the *tkv* defect. A large proportion of these embryos show a mild puckering of the dorsal cuticular surface.

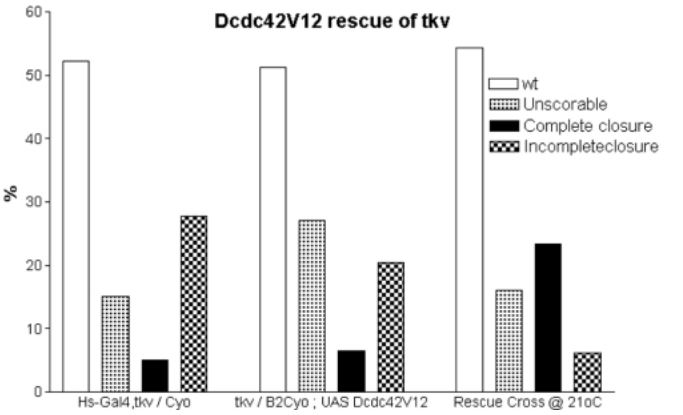

Fig. 6. Bar graph of Dcdc42V12 rescue of the *tkv* phenotype. Embryos were classified as one of four categories: wild-type (Fig. 5A), incomplete closure (Fig. 5B,C) complete closure with mild cuticle defect (Fig. 5D) and unscorable cuticles (empty vitelline membranes or a cuticle too distorted to evaluate. At least 1000 embryos were examined in each experiment. In the parent strain *tkv,HS-GAL4^{2207}* at 25°C (or 21°C, not shown) the proportions of dorsal open (27.7%) and WT (52.2%) embryos were close to the expected one quarter and one half, respectively. The frequency of unscorable embryos was 15.1%, and the frequency with complete closure and mild cuticular defects was 5%. In the other control strain, *tkv/CyO;UAS-Dcdc42V12*, at 25°C the frequencies of embryo with the various categories of phenotype were largely as in *tkv,HS-GAL4^{2207}/CyO* except that the number of unscorable cuticles increased at the expense of wild-type embryos (51.3%), and embryos with incomplete closure (20.5%). In the rescue cross at 21°C (*tkv,HS-GAL4^{2207}/CyO* × *tkv/B2CyO;UAS-Dcdc42V12*) the number of empty cuticles was comparable to *tkv,HS-GAL4^{2207}/Cyo* at 16.1%, while the frequency of embryos displaying *tkv*-like incomplete closure dropped dramatically to 6.2%. The frequencies of wild-type embryos and embryos with complete closure and mild cuticle defects rose to 54.4 % and 23.3%, respectively, indicating a rescue of the DC defect of *tkv*.

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/DJNK 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 anterio-posteriorly directed force which could over constrict 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.

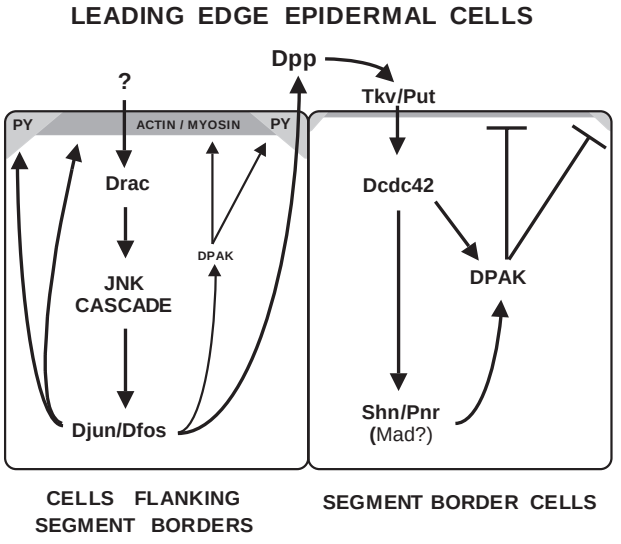

Fig. 7. Participation of Drac1 and Dcdc42 in the JNK and TGF-β cascades to regulate the cytoskeleton during DC. The production of the acto-myosin cytoskeleton, PY-rich focal complexes, and Dpp is set up at the leading edge by a Drac1-mediated JNK cascade. Dpp then binds and activates the TGF-β receptors Tkv and Punt resulting in activation of Dcdc42. The activation of Dcdc42 by the TGF-β receptors then results in the production and/or localisation of DPAK to the leading edge and increased production at the segment borders. LE DPAK, at the segment borders, may transiently downregulate the acto-myosin cytoskeleton and focal complexes to allow the correct dynamics of epidermal migration during DC. Activation of the Thickveins (Tkv) and Punt (Put) receptors sends a signal to the transcription factors Shnurri (Shn) and Pannier (Pnr) which are also required for the presence of elevated DPAK at the LE and the segment borders during DC (see text).

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 mechanisim 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

JNK signaling (Martin-Blanco et al., 1998), and Dpp, and directs assembly of the LE cytoskeleton. Drac1 is also responsible for controlling the normal cell shape changes of the amnioserosa cells 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 pathway via the type-I and type-II TGF-β receptors Punt and Tkv leading to activation of Dcdc42, 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 amnioserosa. We therefore propose the following model for the mechanism of control of DC by the Drac1/JNK and Dcdc42/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 Dcdc42 and the TGF-β pathway, through the S/T 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/Dcdc42 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 Dcdc42/TGFβ 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 Dcdc42 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 Dcdc42-mediated TGF-β signaling, should provide insights into how different signaling pathways can interact to orchestrate epithelial morphogenesis.

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