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Participation of small GTPases in dorsal closure of the *Drosophila* embryo: distinct roles for Rho subfamily proteins in epithelial morphogenesis

Nicholas Harden[1,*], Michael Ricos[2], Yong Mei Ong[1], William Chia[2] and Louis Lim[1,3]

[1]Glaxo-IMCB and [2]Drosophila Neurobiology Laboratories, Institute of Molecular and Cell Biology, 30 Medical Drive, Singapore 117609, Republic of Singapore
[3]Department of Neurochemistry, Institute of Neurology, 1 Wakefield St, London WC1 1PJ, UK
*Author for correspondence at present address: Institute of Molecular Biology and Biochemistry, Simon Fraser University, 8888 University Drive, Burnaby, BC, V5A 1S6, Canada (e-mail: nharden@sfu.ca)

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SUMMARY

The Rho subfamily of Ras-related small GTPases participates in a variety of cellular events including organization of the actin cytoskeleton and signalling by c-Jun N-terminal kinase and p38 kinase cascades. These functions of the Rho subfamily are likely to be required in many developmental events. We have been studying the participation of the Rho subfamily in dorsal closure of the *Drosophila* embryo, a process involving morphogenesis of the epidermis. We have previously shown that Drac1, a Rho subfamily protein, is required for the presence of an actomyosin contractile apparatus believed to be driving the cell shape changes essential to dorsal closure. Expression of a dominant negative Drac1 transgene causes a loss of this contractile apparatus from the leading edge of the advancing epidermis and dorsal closure fails. We now show that two other Rho subfamily proteins, Dcdc42 and RhoA, as well as Ras1 are also required for dorsal closure. Dcdc42 appears to have conflicting roles during dorsal closure: establishment and/or maintenance of the leading edge cytoskeleton versus its down regulation. Down regulation of the leading edge cytoskeleton may be controlled by the serine/threonine kinase DPAK, a potential Drac1/Dcdc42 effector. RhoA is required for the integrity of the leading edge cytoskeleton specifically in cells flanking the segment borders. We have begun to characterize the interactions of the various small GTPases in regulating dorsal closure and find no evidence for the hierarchy of Rho subfamily activity described in some mammalian cell types. Rather, our results suggest that while all Rho subfamily p21s tested are required for dorsal closure, they act largely in parallel.

Key words: *Drosophila*, Rac, Cdc42, Ras, Rho, Small GTPase, Dorsal closure, Cytoskeleton, Morphogenesis

INTRODUCTION

A vast body of work, largely on mammalian cells, has demonstrated the involvement of the Rho subfamily of Ras-related small GTPases (p21s) in a wide range of cellular processes, including regulation of the actin cytoskeleton and signalling through protein kinase pathways (for reviews see Lim et al., 1996; Van Aelst and D'Souza-Schorey, 1997). The Rho subfamily members Rac and Cdc42 bind to and activate the STE20/PAK family of serine/threonine kinases, and also induce signalling through the mitogen-activated protein kinases (MAPKs) p38 and c-Jun N-terminal kinase (JNK). Modification of the actin cytoskeleton and activation of MAPK cascades are likely to be key modulators of development, both in the determination of cell identity and in the movement and morphogenesis of cells. Genetic studies have demonstrated the requirement for signalling by the extracellular signal-regulated kinases (ERKs), which are members of the MAPK family, in a number of developmental events. Ras-activated ERK-signalling is required for the determination of embryonic terminal structures and photoreceptor identity in *Drosophila*, and vulval development in *C. elegans* (reviewed by Dickson and Hafen, 1994).

The Rho subfamily members have essential roles in development. Drac1 participates in myoblast fusion and the outgrowth and guidance of axons during *Drosophila* embryogenesis (Luo et al., 1994; Kaufmann et al., 1998) and, in the wing disc epithelium, is involved in the recruitment of actin to adherens junctions and hair outgrowth (Eaton et al., 1995, 1996). Expression of RacV12 in the Purkinje cells of transgenic mice affects axons and dendritic spines (Luo et al., 1996). Dcdc42, a *Drosophila* Cdc42 homologue, is required for the proper outgrowth of dendrites and axons (Luo et al., 1994), is involved in determining the shape of both muscle fibres (Luo et al., 1994) and wing disc epithelial cells (Eaton et al., 1995), and participates in wing hair outgrowth (Eaton et al., 1996). Dcdc42, Drac1, and a new member of the Rho subfamily, RhoL, have cell type-specific functions in *Drosophila* oogenesis (Murphy and Montell, 1996). *Drosophila* RhoA (Rho1) regulates tissue polarity (Strutt et al.,

1997), participates in gastrulation (Barrett et al., 1997) and its overexpression can cause a defect late in eye development (Hariharan et al., 1995).

We are interested in understanding how the various cellular roles of the Rho subfamily contribute to tissue morphogenesis during development. We have previously reported a role for Rac in dorsal closure (DC), a morphogenetic process occurring during *Drosophila* embryogenesis (Harden et al., 1995). Following germband retraction, a hole is left in the dorsal epidermis of the embryo which is occupied by the large, flat cells of the amnioserosa. Beginning at stage 14 there is a dorsally directed movement of the lateral epidermis from both sides of the embryo. The two migrating flanks of the epidermis move over the amnioserosa and meet up along the dorsal midline, completely sealing the hole by stage 15 (Campos-Ortega and Hartenstein, 1985). During DC the leading edge of the advancing epidermis, comprised of the dorsal most ends of the epidermal cells flanking the amnioserosa, shows a dramatic accumulation of filamentous actin (F-actin) and nonmuscle myosin heavy chain (hereafter referred to as myosin). The leading edge cells undergo an elongation along the dorsoventral axis, and as DC proceeds a similar elongation is seen in more ventrally located epidermal cells. It has been proposed that the accumulation of F-actin and myosin at the leading edge forms an actomyosin contractile apparatus driving the elongation of the leading edge cells (Young et al., 1993). More ventrally located epidermal cells are then passively elongated as a result of the contractions at the leading edge, and DC proceeds through a stretching of the epidermis over the amnioserosa. We have shown that expression of dominant negative Rac causes a failure of DC, accompanied by impaired epidermal cell elongation and a loss of both F-actin and myosin from the leading edge (Harden et al., 1995). We have also demonstrated that phosphotyrosine-rich nodes and DPAK, a member of the PAK family, are found along the leading edge, and that these leading edge components are lost following dominant negative Rac expression (Harden et al., 1996). In addition to Rac, members of a JNK signalling pathway (Glise et al., 1995; Riesgo-Escovar et al., 1996; Sluss et al., 1996; Glise and Noselli, 1997; Hou et al., 1997; Riesgo-Escovar and Hafen, 1997; Kockel et al., 1997) are required for DC. Thus, DC is an excellent system for analyzing the integration of Rho subfamily-mediated cytoskeletal regulation and signal transduction. The process of DC is presented schematically in Fig. 1.

In this study, we extend our characterization of the participation of small GTPases in DC. We show that all Rho-subfamily members tested, and Ras1, are required for DC. We have found that Drac1 is essential for the formation and/or maintenance of the cytoskeleton all along the leading edge, whereas RhoA function is required for the leading edge cytoskeleton specifically in groups of cells flanking the segment borders. Dcdc42 and Ras1 appear to be involved more in regulation of the leading edge cytoskeleton. We have begun to address the issue of how the different p21s interact with each other through co-expression studies. Most importantly, our data indicate that the hierarchy of Rho subfamily activation described in fibroblasts (Nobes and Hall, 1995) and macrophages (Allen et al., 1997) does not occur in the leading edge cells during DC.

MATERIALS AND METHODS

Molecular biological techniques were performed using standard procedures (Sambrook et al., 1989).

Fly strains

HA-tagged wild-type, activated and dominant negative versions of a human RhoA cDNA (Leung et al., 1995, 1996) were excised from the pXJ40 vector through an *Eco*RI/*Bgl*II digestion and cloned into the *Eco*RI and *Bgl*II sites of the vector pUAST (Brand and Perrimon, 1993). The RhoA pUAST constructs were injected into *Df(1)yw* embryos as described by O'Connor and Chia (1993) and transgenic lines established. Flies bearing dominant negative and activated forms of *Drac1* and *Dcdc42* in pUAST were from L. Luo (Luo et al., 1994), dominant negative *Drosophila* RhoA in pUAST from M. Mlodzik (Strutt et al., 1997), dominant negative Ras1 in pUAST from T. Lee (Lee et al., 1996), and activated Ras1 in pUAST (*UAS-Ras1Q13(II)B)* from B. Noll. The various p21s were expressed using the GAL4 system of Brand and Perrimon (1993). Females from GAL4 lines were crossed to males from the pUAST transgenic lines and the progeny examined as embryos. The GAL4 lines *GAL4*$^{559.1}$, *Hs-GAL4*$^{M-4}$, and *Hs-GAL4*^{2077} were provided by E. Knust, J. Roote, and the Bloomington Stock Center, respectively. Expression of the human

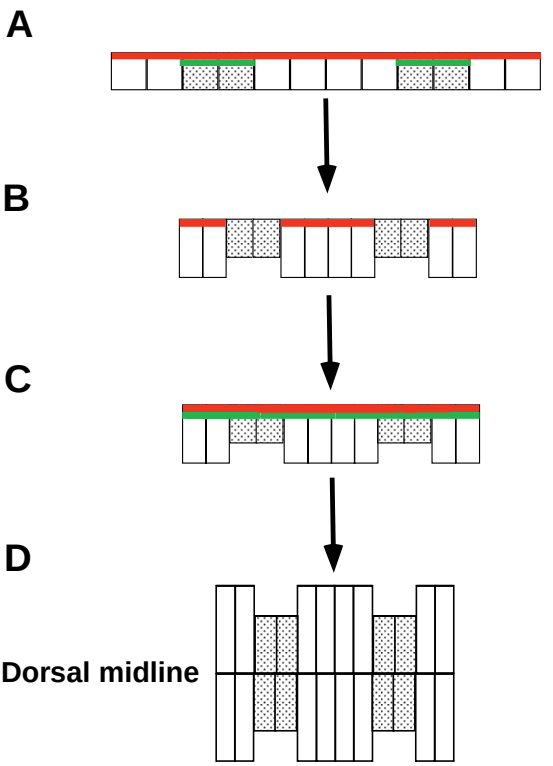

Fig. 1. Schematic diagram of the leading edge during dorsal closure (DC) illustrating features of interest to this study. Red lines represent leading edge F-actin and myosin, green lines leading edge DPAK, segment border cells are shaded. (A-C) Lateral views (D) a dorsal view. (A) At the beginning of DC F-actin and myosin are present along the leading edge and DPAK levels are high in the segment border cells. (B) During DC there is transient loss of the leading edge cytoskeleton in segment border cells, and this is the likely reason for these cells remaining less elongated than their neighbours. (C) Later in DC there are no gaps in the leading edge cytoskeleton, as cytoskeletal losses in the segment border cells are transient. DPAK levels are now elevated all along the leading edge. (D) At the end of DC the leading edge cells from each side of the embryo meet along the dorsal midline and the leading edge cytoskeleton is disassembled.

RhoA transgenes was confirmed by crossing males from the various transgenic lines to *Hs-GAL4^{M-4}* females and testing their progeny for heat-shock induction of the HA tag by western analysis (data not shown).

Flies bearing dominant negative *DRacA* under heat-shock control (line N17-104) were as previously described (Harden et al., 1995). For the sake of consistency in nomenclature, we have renamed this line *Hs-Drac1N17-104*, as the *DRacA* gene is the same gene as *Drac1* (Luo et al., 1994). Flies bearing an activated version of *Drosophila Ras1* in the pCaSpeR-hs vector, *Hs-Ras1Q13-9.4-M12* (Lu et al., 1993) were a gift from B. Noll.

Heat-shock expression of transgenes

Unless otherwise stated, the following protocols were used. Embryos were collected and aged at 25°C until 8 to 12 hours after egg laying (AEL). They were then placed in vials and heat-shocked for 1 hour in a water bath set at 37°C. Following heat-shock, embryos were either aged at 21°C for at least 48 hours and subjected to cuticle preparation, or aged for 7 hours at 21°C and fixed for immunohistochemistry. Control embryos were collected from all crosses and were maintained at 25°C prior to cuticle preparation or immunohistochemistry.

Immunohistochemistry

All procedures were carried out at room temperature, unless otherwise stated. Embryos were dechorionated in 50% household bleach in 0.01% Triton X-100, washed in 0.01% Triton and fixed for 25 minutes in 4% paraformaldehyde in PBS (0.1 M NaCl, 10 mM phosphate buffer, pH 7.4)/heptane. Vitelline membranes were removed by washing with methanol, or 80% ethanol if embryos were to be phalloidin stained. Embryos were washed for one hour in several changes of PBT (PBS with 0.1% Triton X-100), and blocked for one hour in PBT containing 1% bovine serum albumin (BSA). Primary antibody incubations were done overnight at 4°C in PBT containing 1% BSA. Following overnight incubation, embryos were washed for one hour in PBT. Fluorescent detection of primary antibodies was done using either biotinylated secondary antibodies and streptavidin labelled with Texas Red or FITC, or secondary antibodies directly labelled with Texas Red or FITC (all materials from Vector Laboratories). All secondary antibodies were diluted 1:200 in 1% BSA in PBT. Secondary antibody incubation was done for two hours. Embryos were then washed for one hour in several changes of PBT and incubated with a 1:1000 dilution of labelled streptavidin in PBS for one hour. When F-actin staining was required, FITC-labelled or TRITC-labelled phalloidin (Sigma) was added to a final concentration of 1 μg/ml half an hour into the streptavidin incubation. Embryos were washed for 20 minutes in several changes of PBS, mounted in Vectashield and viewed on a Bio-Rad MRC 600 confocal laser scanning microscope.

Cuticle preparations

Cuticles were prepared as described by Ashburner (1989), but with the fixation step removed. At least 100 embryos were examined in each experiment.

RESULTS

Expression of Drac1N17 using *patched* (*ptc*) promoter-driven GAL4 causes DC defects similar to heat-shock expression

We wondered to what degree the leading edge effects and DC defects caused by heat-shock induction of Drac1N17 (Harden et al., 1995, 1996) might be due to expression of Drac1N17 in the amnioserosa, as this tissue is a likely source of inductive signals regulating the leading edge, and disruption of amnioserosa morphology can cause a pronounced DC defect (Wodarz et al., 1995). In order to exclude Drac1 from the amnioserosa, we expressed various UAS-coupled *Drac1* transgenes (Luo et al., 1994) using GAL4 driven by the *patched* (*ptc*) promoter (*GAL4$^{559.1}$*; Hinz et al., 1994). Prior to the commencement of DC, in the stage 10 embryo, *ptc* is widely expressed in the ectoderm, occupying the posterior three-quarters of each parasegment (Nakano et al., 1989). By stage 12 this expression is reduced to two narrow stripes per segment, and this pattern is maintained throughout DC. There are no detectable *ptc* transcripts in the amnioserosa.

A *UAS-Drac1N17* transgene was expressed using the *GAL4$^{559.1}$* driver or by heat-shock using the *Hs-GAL4^{M-4}* driver. In both cases, embryos showed a cuticle phenotype very similar to that seen with heat-shock inductions of *Drac1N17* cloned into the pCaSpeR-hs vector (Harden et al., 1995), and had dorsal holes ranging in size from a small 'scab' to a wide open dorsal surface (Fig. 2A). *Hs-GAL4^{M-4};UAS-Drac1N17* embryos exhibited complete or almost complete losses of, myosin, F-actin and phosphotyrosine nodes from the leading edge (data not shown). In *GAL4$^{559.1}$;UAS-Drac1N17* embryos, these cytoskeletal components were similarly lost from the leading edge. However, patches of all three were found at regular intervals along the leading edge, extending from the middle of segments towards the posterior of segments. (Fig. 3D-G). These regions of undisrupted leading edge structure coincided well with areas that would not have expressed *ptc* earlier in development, and thus would have escaped Drac1N17 expression prior to DC. Double labelling studies indicated that where one of the leading edge components persisted, the others also remained (data not shown). Examination of phosphotyrosine-labelled embryos revealed that those cells which retained the leading edge cytoskeleton were narrower along the anterior-posterior axis than their neighbours (Fig. 3D,E).

To see the effects on DC of excessive Drac1 signalling, we expressed a constitutively active version of Drac1, Drac1V12, using the *GAL4$^{559.1}$* and *Hs-GAL4^{M-4}* drivers. Expression of Drac1V12 with either driver had a drastic effect on the embryonic cuticle, with most embryos producing only small pieces of cuticle. Stained embryos exhibited a dramatic bunching of the lateral epidermis around the amnioserosa and it was not possible to evaluate the effects of Drac1V12 on the leading edge (N. Harden and M. Ricos, unpublished observations).

Dcdc42N17 disrupts the accumulation of cytoskeletal elements and DPAK at the leading edge and can cause misregulated contraction of the lateral epidermis

Previous studies in *Drosophila* have indicated that the closely related molecules Drac1 and Dcdc42, while participating in shared developmental events, have separable functions (Luo et al., 1994; Eaton et al., 1995, 1996; Murphy and Montell, 1996). To determine if Dcdc42, like Drac1, participates in DC, dominant negative Dcdc42N17 was expressed using the same drivers as with Drac1N17. There is a high frequency of dorsal cuticle defects among *GAL4$^{559.1}$;UAS-Dcdc42N17* embryos. Unlike *GAL4$^{559.1}$;UAS-Drac1N17* embryos, which show considerable variation in the size of the dorsal hole, the dorsal phenotype of *GAL4$^{559.1}$;UAS-Dcdc42N17* embryos is more

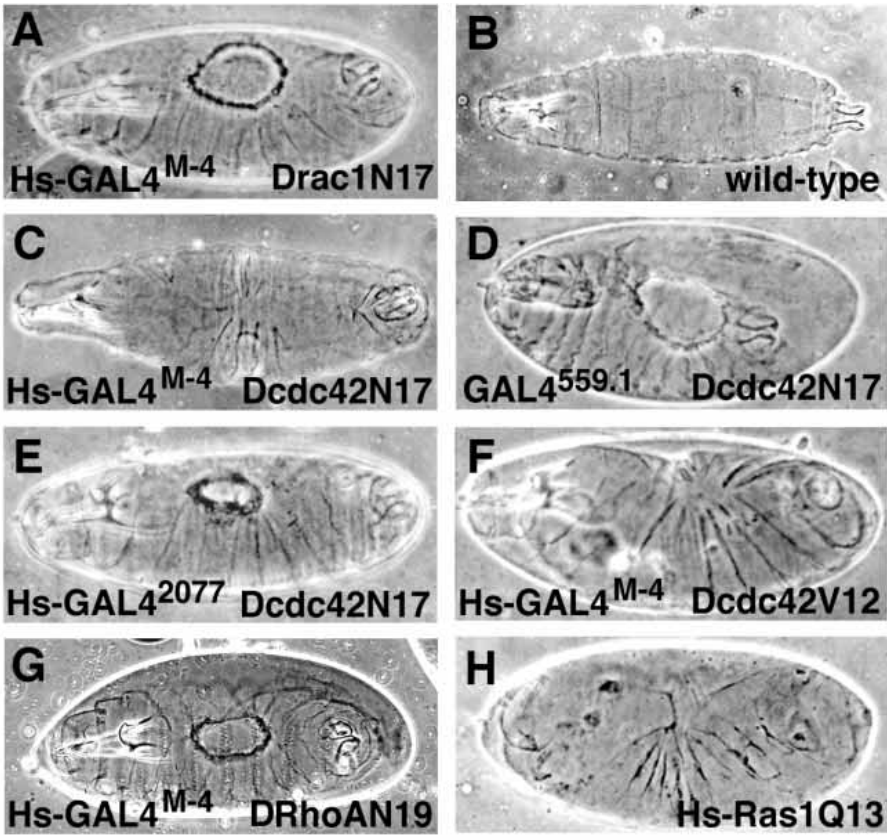

Fig. 2. Effects of p21 transgene expression on the dorsal cuticle. Dorsolateral (A,D-F,H) or dorsal (B,C,G) views of cuticle preparations of embryos and first instar larvae. Anterior is left. (A) *Hs-GAL4^{M-4};UAS-Drac1N17* embryo showing hole in dorsal cuticle. (B) Wild-type first instar larva. (C) *Hs-GAL4^{M-4}; UAS-Dcdc42N17* first instar larva with mild dorsal pucker. (D) *GAL4$^{559.1}$;UAS-Dcdc42N17* embryo with large dorsal hole towards posterior. (E) *Hs-GAL4^{2077};UAS-Dcdc42N17* embryo showing hole in dorsal cuticle. (F) *Hs-GAL4^{M-4};UAS-Dcdc42V12* embryo with pronounced puckering of dorsal cuticle. (G) *Hs-GAL4^{M-4};UAS-DRhoAN19* embryo with dorsal hole. (H) *Hs-Ras1Q13-9.4-M12* embryo showing strong dorsal pucker.

uniform, with most showing a large hole towards the posterior of the embryo (Fig. 2D). Many *Hs-GAL4^{M-4};UAS-Dcdc42N17* embryos survived to the first instar larval stage, and phenotypes were generally limited to a mild puckering of the cuticle, with very few dorsal holes occurring (Fig. 2C). During the course of this work Riesgo-Escovar et al. (1996) reported that expression of *UAS-Dcdc42N17* using the epidermal *GAL4-69B* driver caused a DC defect. This DC defect is very similar to what we see in *GAL4$^{559.1}$;UAS-Dcdc42N17* embryos.

Embryos in which *UAS-Dcdc42N17* had been induced with the *GAL4$^{559.1}$* driver or by heat-shock with the *Hs-GAL4^{M-4}* driver were fixed and stained for F-actin, myosin, and phosphotyrosine. With both drivers partial losses of these leading edge components were seen as compared to control embryos (Fig. 3H-J), although many embryos had levels of leading edge components comparable to controls (data not shown). *GAL4$^{559.1}$;UAS-Dcdc42N17* embryos showed persistence of leading edge components in the same segmental regions as seen in *GAL4$^{559.1}$;UAS-Drac1N17* embryos. Elsewhere along the leading edge, *GAL4$^{559.1}$;UAS-Dcdc42N17* embryos showed decreases of leading edge components as compared with control embryos, but did not exhibit the complete or near complete losses seen in the corresponding regions of *GAL4$^{559.1}$;UAS-Drac1N17* embryos (data not shown). These results indicate that Dcdc42N17 can only cause a partial loss of the leading edge cytoskeleton in regions where it is expressed and that its effects are clearly less severe than those produced by Drac1N17.

During DC, the putative Drac1/Dcdc42 effector DPAK becomes enriched in the leading edge epidermal cells (Harden et al., 1996; shown schematically in Fig. 1). In the early stages of DC several cells flanking each segment border (Fig. 4A) show elevated DPAK staining. As DC proceeds high levels of DPAK appear all along the leading edge (Fig. 4G). In HeLa cells, mammalian α-PAK is recruited to focal complexes by constitutively active Cdc42 (Manser et al., 1997), and we wondered if Dcdc42 would contribute to DPAK localization. To examine this, we stained *GAL4$^{559.1}$;UAS-Dcdc42N17* and *Hs-GAL4^{M-4};UAS-Dcdc42N17* embryos with anti-DPAK (Harden et al., 1996). It is difficult to evaluate DPAK levels at the leading edge in heat-shocked *Hs-GAL4^{M-4};UAS-Dcdc42N17* embryos due to elevated levels of DPAK staining in the amnioserosa (Fig. 4B). This Cdc42N17-induced increase in DPAK levels will be the subject of a future communication. *GAL4$^{559.1}$;UAS-Dcdc42N17* embryos do not exhibit DPAK elevation in the amnioserosa and the leading edge can be evaluated. Late in DC many are found to be lacking the normal accumulation of DPAK along the leading edge (Fig. 4H), despite often having near normal levels of phosphotyrosine (Fig. 4I). This is in contrast to our earlier results with Drac1N17, where DPAK is only lost from the leading edge in those areas severely depleted for the other leading edge components (Harden et al., 1996).

We have expressed *UAS-Dcdc42* and *UAS-Drac1* transgenes using a second heat-shock GAL4 driver, *Hs-GAL4^{2077}*. Based on the phenotypes caused by *UAS-Drac1* transgene expression we have determined that *Hs-GAL4^{2077}* is a weaker driver than *Hs-GAL4^{M-4}* (data not shown). Surprisingly, expression of *UAS-Dcdc42N17* with *Hs-GAL4^{2077}* produced a greater number of dorsal holes than with *Hs-GAL4^{M-4}* (Fig. 2E). To understand how a weaker driver could produce a more severe defect, we examined *Hs-GAL4^{2077};UAS-Dcdc42N17* embryos further. *Hs-*

GAL4^{2077};UAS-Dcdc42N17 embryos stained for phosphotyrosine exhibited a dramatic change in the lateral epidermis at the leading edge. Most embryos had at least one and usually several instances of the dorsal ends of segments being pulled together at the leading edge into bunches (Fig. 5A,B). In many cases it appeared that adhesions had formed between cells brought into apposition by this bunching (arrowheads in Fig. 5A,B). The bunching of the epidermis, rarely seen with *Hs-GAL4^{M-4}*, is likely to be the reason for the serious DC defects seen in cuticle preparations of *Hs-GAL4^{2077};UAS-Dcdc42N17* embryos, as it prevents proper sealing of the two leading edges at the end of DC (data not shown). Examination of the leading edge cytoskeleton is difficult in these embryos due to the bunching. The overall impression gained from examining *Hs-GAL4^{2077};UAS-Dcdc42N17* embryos is that the bunches represent regions of excessive contraction of the leading edge.

Dcdc42V12 can result in increased DPAK at the leading edge but can also cause loss of leading edge components, including DPAK

We have assessed the effects on DC of excessive Dcdc42 signalling by expressing a constitutively active version of Dcdc42, Dcdc42V12, using the same drivers as in the above studies. Most of the cuticles of *GAL4$^{559.1}$;UAS-Dcdc42V12* embryos were either badly distorted or missing, whereas *Hs-GAL4^{M-4};UAS-Dcdc42V12* and *Hs-GAL4^{2077};UAS-Dcdc42V12* embryos exhibited a high frequency of puckers in the dorsal surface (Fig. 2F). These puckers were more severe than in *Hs-GAL4^{M-4};UAS-Dcdc42N17* embryos, and very few of these individuals hatched.

Examination of fixed embryos in which *UAS-Dcdc42V12* expression had been induced with either the *GAL4$^{559.1}$* or *Hs-GAL4^{M-4}* driver, revealed an extremely varied effect on leading edge components. F-actin, myosin and phosphotyrosine levels ranged from complete absence to normal levels, while DPAK staining ranged from complete absence to dramatic elevation. As most *GAL4$^{559.1}$;UAS-Dcdc42V12* embryos were badly distorted we restricted further analysis of UAS-Dcdc42V12-induced phenotypes to *Hs-GAL4^{M-4};UAS-Dcdc42V12* embryos. While control embryos early in DC show heavy leading edge DPAK staining only at the segment borders (Fig. 4A), about 50% of *Hs-GAL4^{M-4};UAS-Dcdc42V12* embryos at this stage show heavy DPAK staining extending along the leading edge beyond the segment borders (Fig. 4C), a DPAK distribution that

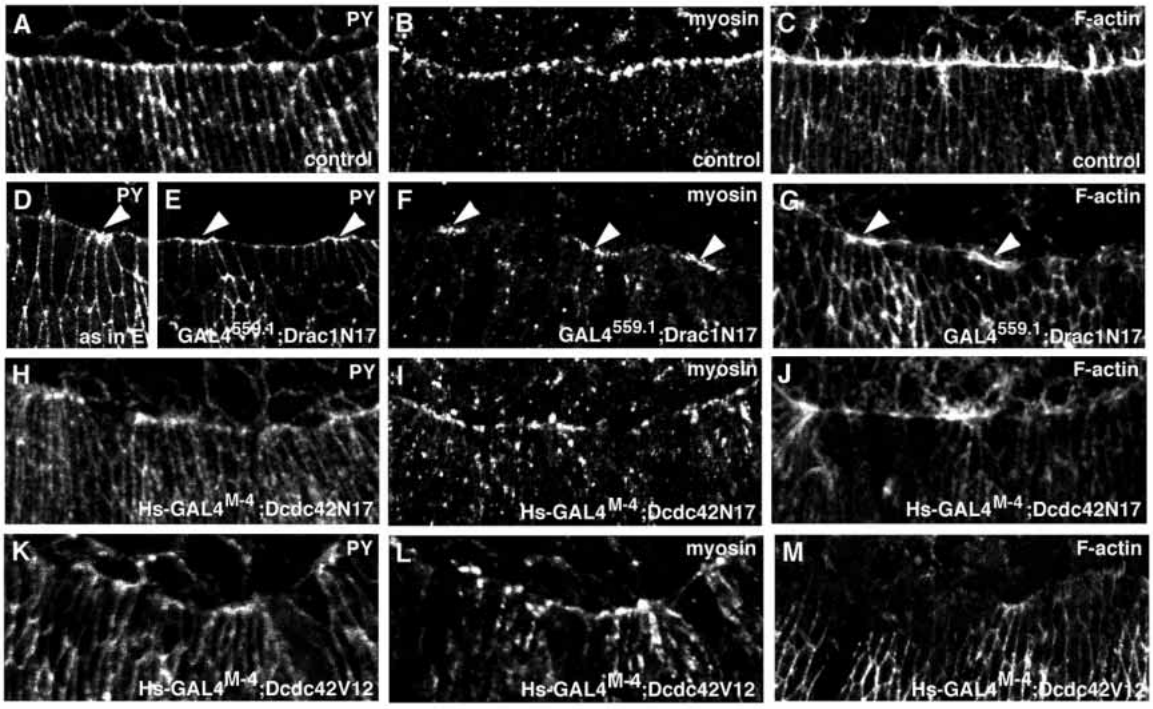

Fig. 3. Effects on the leading edge cytoskeleton of expressing various p21 transgenes. Confocal fluorescent micrographs show the boundary between the amnioserosa (top of each micrograph) and the epidermis during early stages of DC. Anterior is to the left in this and subsequent figures. Embryos were stained with anti-phosphotyrosine antibodies, anti-nonmuscle myosin antibodies (Kiehart and Feghali, 1986), or phalloidin to detect F-actin. (A) Control staining of unheat-shocked *Hs-GAL4^{M-4};UAS-Dcdc42N17* embryo showing triangular nodes of phosphotyrosine (PY) staining along the leading edge. (B) Control staining of unheat-shocked *Hs-GAL4^{M-4};UAS-Dcdc42N17* embryo showing punctate distribution of myosin along the leading edge. (C) Control staining of unheat-shocked *Hs-GAL4^{M-4};UAS-Dcdc42V12* embryo showing F-actin accumulation along the leading edge. (D,E) *GAL4$^{559.1}$;UAS-Drac1N17* embryo showing a segmentally reiterated pattern of patches of phosphotyrosine (arrowheads). (F) *GAL4$^{559.1}$;UAS-Drac1N17* embryo showing a segmentally reiterated pattern of patches of myosin (arrowheads). (G) *GAL4$^{559.1}$;UAS-Drac1N17* embryo showing a segmentally reiterated pattern of patches of F-actin (arrowheads). (H) *Hs-GAL4^{M-4};UAS-Dcdc42N17* embryo showing partial loss of leading edge phosphotyrosine nodes. (I) *Hs-GAL4^{M-4};UAS-Dcdc42N17* embryo showing partial loss of leading edge myosin. (J) *Hs-GAL4^{M-4};UAS-Dcdc42N17* embryo showing partial loss of leading edge F-actin. (K) *Hs-GAL4^{M-4};UAS-Dcdc42V12* embryo showing loss of leading edge phosphotyrosine nodes. (L) *Hs-GAL4^{M-4};UAS-Dcdc42V12* embryo showing loss of leading edge myosin. (M) *Hs-GAL4^{M-4};UAS-Dcdc42V12* embryo showing loss of leading edge F-actin. An anti-DPAK staining of the same embryo is shown in Fig. 4D.

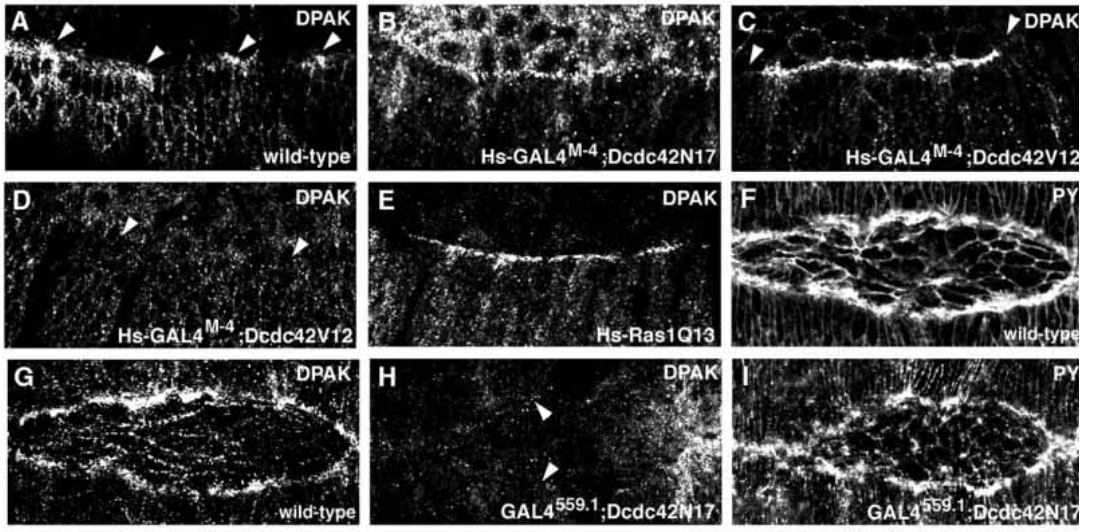

Fig. 4. Effects of p21 transgene expression on leading edge DPAK staining. Embryos were stained with anti-DPAK antibodies (Harden et al., 1996) or anti-phosphotyrosine antibodies. (A) Wild-type embryo early in DC showing elevated DPAK at the leading edge in cells flanking the segment borders. (B) *Hs-GAL4^{M-4};UAS-Dcdc42N17* embryo showing elevated DPAK in the amnioserosa. (C) *Hs-GAL4^{M-4};UAS-Dcdc42V12* embryo early in DC showing elevated DPAK at the leading edge as compared to the control embryo in A. Arrowheads show patches where DPAK is absent. A phalloidin staining of the same embryo shows loss of leading edge F-actin in the same regions as those deficient in leading edge DPAK (data not shown). (D) *Hs-GAL4^{M-4};UAS-Dcdc42V12* embryo showing lack of DPAK along the leading edge (arrowheads). A phalloidin staining of the same embryo is shown in Fig. 3M. (E) *Hs-Ras1Q13-9.4-M12* embryo early in DC showing elevated DPAK at the leading edge as compared to the control embryo in A. (F) Wild-type embryo late in DC showing strong phosphotyrosine staining along the leading edge. (G) Same embryo as in F, stained to show DPAK accumulation along the leading edge. (H) *GAL4$^{559.1}$;UAS-Dcdc42N17* embryo late in DC, showing lack of DPAK along the leading edge (arrowheads). (I) Same embryo as in H, showing phosphotyrosine staining along the leading edge.

normally occurs only later in DC (Fig. 4G). Leading edge levels of F-actin in these *Hs-GAL4^{M-4};UAS-Dcdc42V12* embryos are comparable to controls (data not shown). Interestingly, patches of leading edge can be found in these embryos that are deficient in both DPAK and F-actin (Fig. 4C, arrowheads, data not shown). About 10% of *Hs-GAL4^{M-4};UAS-Dcdc42V12* embryos are lacking in DPAK all along the leading edge, and this deficiency is accompanied by severe reductions in the amount of leading edge F-actin (Figs 3M, 4D). Similarly, about 10% of *Hs-GAL4^{M-4};UAS-Dcdc42V12* embryos double stained for phosphotyrosine and myosin have deficiencies of these components at the leading edge (Fig. 3K,L). We have looked to see if the frequency of embryos missing DPAK and other leading edge components can be higher than 10% at any time after the heat-shock induction of *UAS-Dcdc42V12*. Embryos were fixed at half hour intervals between 6 and 8.5 hours after *UAS-Dcdc42V12* induction and stained for F-actin and DPAK, but no increase in the frequency of leading edge deficient embryos was seen (data not shown). By 8.5 hours post heat-shock, neither elevated DPAK levels nor embryos deficient in F-actin/DPAK were seen.

Drosophila RhoAN19 causes loss of the leading edge cytoskeleton in cells flanking the segment borders

We first investigated the potential involvement of Rho proteins in DC by expressing human RhoA transgenes during embryogenesis. Previous studies have indicated that expression or injection of dominant negative or activated forms of mammalian p21s can disrupt signalling by their *Drosophila* homologues (Lu et al., 1993; Lee et al., 1996). Injection of dominant negative human RhoA into cellularizing embryos results in the same phenotype as injection of C3 exoenzyme from *Clostridium botulinum* (Crawford et al., 1998). C3 exoenzyme is a Rho-specific inhibitor that has been widely used to characterize Rho-function (reviewed by Narumiya et al., 1997), These data indicate that dominant negative human RhoA is disrupting Rho function in the embryo.

When *UAS-RhoAN19* or *UAS-RhoAV14* were expressed by heat-shock using the *Hs-GAL4^{M-4}* driver, cuticle preparations revealed many first instar larvae with dorsal puckers (data not shown). These mild DC defects are very similar to those seen in *Hs-GAL4^{M-4};UAS-Dcdc42N17* individuals (Fig. 2C). During the course of this study, Strutt et al. (1997) reported the isolation of mutants in the *Drosophila RhoA* (*Rho1*) gene. Interestingly, null alleles of *Drosophila RhoA* can cause DC defects. Strutt et al. (1997) have made transgenic flies bearing a dominant negative version of *Drosophila RhoA* in the pUAST vector. Expression of this transgene, *UAS-DRhoAN19*, in embryos using the *Hs-GAL4^{M-4}* driver also causes a high frequency of dorsal defects, although they tend to be more severe than those induced by the human *RhoA* transgenes, as many embryos have holes in the dorsal surface (Fig. 2G).

Mutant human *RhoA* transgene expression produced no discernible effect on the leading edge distribution of F-actin, phosphotyrosine, myosin or DPAK (data not shown). However, embryos expressing any one of the mutant *RhoA* transgenes show some bunching of the lateral epidermis and their dorsal holes tend to be considerably larger than control embryos of similar age, indicating that DC is impaired (data not shown). Heat-shock expression of *UAS-DRhoAN19* has a dramatic effect on the leading edge cytoskeleton, as visualized by staining for myosin (Fig. 5C). There is a segmentally repeated loss of myosin from the leading edge at each segment boundary. Myosin levels elsewhere along the leading edge appear normal. When *Hs-GAL4^{M-4};UAS-DRhoAN19* embryos are stained for phosphotyrosine, the same pattern of loss is seen, with phosphotyrosine nodes being lost from the leading

edge in groups of between two and four cells flanking the segment borders, and leading edge phosphotyrosine nodes elsewhere being unaffected (Fig. 5D). Embryos fixed in the later stages of dorsal closure show anterior-posterior contraction of those cells with phosphotyrosine nodes, while the segment border cells are not contracted and tend to be splayed out (Fig. 5D). The overall effect is an unevenly contracted leading edge distinct from the uneven leading edge contractions generated by Cdc42N17 expression (Fig. 5A,B).

Ras1N17 can cause partial losses of the leading edge cytoskeleton

Evidence from work on mammalian cells indicates that the Rho subfamily is involved in the cytoskeletal changes and transformation mediated by Ras (reviewed by Symons, 1996). Therefore, we wondered if Ras might be required for DC. A dominant negative version of *Drosophila* Ras1 cloned into the pUAST vector, *UAS-Ras1N17* (Lee et al., 1996), was expressed during embryogenesis using the *Hs-GAL4^{M-4}* driver and the embryonic cuticles examined. Expression of Ras1N17 resulted in many first instar larvae with mild puckering of the dorsal cuticle, a phenotype very similar to those generated by Dcdc42N17 and the human *RhoA* transgenes (data not shown). Many *Hs-GAL4^{M-4};UAS-Ras1N17* embryos exhibited partial losses of F-actin, myosin, and phosphotyrosine from the leading edge, while DPAK levels remained unaffected (data not shown). The partial losses of leading edge cytoskeletal components were reminiscent of these seen in *Hs-GAL4^{M-4};UAS-Dcdc42N17* embryos.

Ras1Q13 expression can result in increased DPAK at the leading edge

We have characterized the effects of excessive Ras1 signalling in DC using two transgenes bearing constitutively active Ras1(Ras1Q13): *UAS-Ras1Q13(II)B* and *Hs-Ras1Q13-9.4-M12* (Lu et al., 1993). Expression of Ras1Q13 by either heat-shock or using the *GAL4$^{559.1}$* driver resulted in a high frequency of defects in the dorsal cuticle (Fig. 2H). For both forms of Ras1Q13 induction there was, relative to controls, a clear increase in DPAK at the leading edge of many embryos in the early stages of DC. As with Dcdc42V12, this increase was in the form of a premature accumulation of DPAK all along the leading edge at a developmental stage when DPAK is normally elevated only in the segment border cells (Fig. 4E, compare to Fig. 4A); this increase in leading edge DPAK caused by Ras1Q13 expression was generally weaker than that resulting from Dcdc42V12 expression. Unlike the Dcdc42V12 inductions, there were no deficiencies in leading edge F-actin, myosin, phosphotyrosine or DPAK following Ras1Q13 expression in embryos. Many embryos in which Ras1Q13 had been expressed exhibited a bunching of the lateral epidermis similar to that seen in *Hs-GAL4^{2077};UAS-Dcdc42N17* embryos, although the bunching tended to be less severe and occurred at a lower frequency (data not shown).

The frequency of Drac1N17-induced DC defects can be reduced by co-expression with *Dcdc42* transgenes

Given the evidence of a Rho subfamily hierarchy of p21 activity, we were interested in looking at the interactions among the Rho subfamily p21s participating in DC. We began by evaluating the effects of expressing the various p21 transgenes on the leading edge losses and DC failure caused by *Hs-Drac1N17-104*.

As expected Drac1V12 was able to override the Drac1N17 phenotype and *Hs-GAL4^{M-4};Hs-Drac1N17-104;UAS-Drac1V12* embryos exhibited a range of phenotypes similar to *Hs-GAL4^{M-4};UAS-Drac1V12* embryos (data not shown). We next evaluated the effects on the Drac1N17 phenotype of expressing dominant negative or constitutively active Dcdc42 (Table 1). Cuticle preparations indicate that if Drac1N17 and Dcdc42N17 are co-expressed before the commencement of DC (4 to 8 hours AEL), the DC phenotype is more severe, both in terms of numbers and type of defects, than that seen when either transgene is expressed alone at this time. In contrast, if Drac1N17 and Dcdc42N17 are co-expressed during DC (8 to 12 hours AEL) the DC phenotype is weaker than Drac1N17 expressed alone but stronger than Dcdc42N17 expressed alone. Thus, there is a temporal shift from Dcdc42N17 worsening the effects of Drac1N17 to partially rescuing the effects of Drac1N17.

When Dcdc42V12 was co-expressed with Drac1N17, most of the resulting cuticles were too severely disrupted to evaluate, and we have only examined cuticles of embryos in which the transgenes were induced 10 to 12 hours AEL. Even in this case, 60% of the cuticles are uninterpretable. However, there is evidence of some rescue of the DRac1N17 phenotype by Dcdc42V12, as there are greater numbers of wild-type and

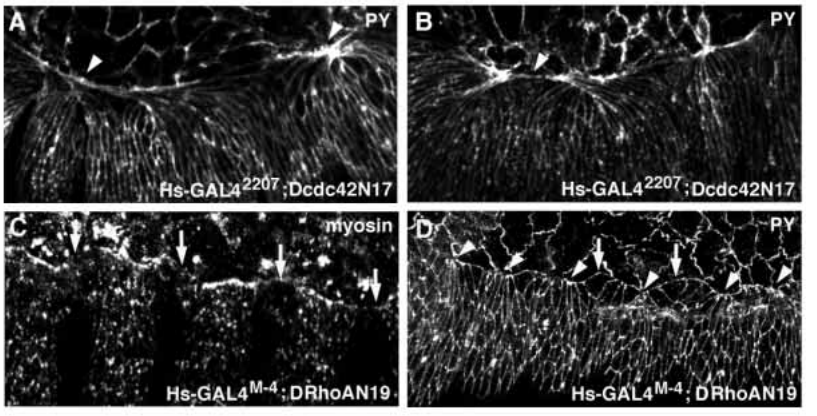

Fig. 5. Dcdc42N17 and DRhoAN19 both cause abnormal contractions of the leading edge but the phenotypes are distinct. (A,B) *Hs-GAL4^{2077};UAS-Dcdc42N17* embryos stained with anti-phosphotyrosine antibodies, showing dramatic bunching of segments at the leading edge. Arrowheads indicate adhesion of cells brought together by bunching. (C) *Hs-GAL4^{M-4};UAS-DRhoAN19* embryo stained for myosin, showing loss of leading edge myosin in cells flanking the segment borders (arrows). (D) *Hs-GAL4^{M-4};UAS-DRhoAN19* embryo at a later stage of closure than that in C, stained for phosphotyrosine. Cells flanking segment borders lack leading edge phosphotyrosine nodes and are splayed out, not showing the normal anterior-posterior contraction. Arrows indicate two examples of splayed cells, each in a group of four such segment border cells. Elsewhere along the leading edge, phosphotyrosine nodes are present and cells are contracted in the anterior-posterior axis (arrowheads). These cells form segmentally reiterated patches of leading edge contraction.

Table 1. Effects of co-expression of Drac1 and Dcdc42 transgenes on dorsal cuticle phenotype frequencies

	Pucker in dorsal cuticle (%)	Hole in dorsal cuticle (%)	Dorsal cuticle unscoreable (%)	Wild type dorsal cuticle (%)
Hs-GAL4^{M-4};Hs-Drac1N17-104				
4 to 8 hours AEL*	14	36	33	17
8 to 12 hours AEL	16	51	14	19
Hs-GAL4^{M-4};UAS-Dcdc42N17				
4 to 8 hours AEL	34	2	37	27
8 to 12 hours AEL	46	1	17	36
Hs-GAL4^{M-4};Hs-Drac1N17-104; UAS-Dcdc42N17				
4 to 8 hours AEL	8	48	35	9
8 to 12 hours AEL	18	32	16	38
Hs-GAL4^{M-4};Hs-Drac1N17-104				
10 to 12 hours AEL	10	65	16	9
Hs-GAL4^{M-4};UAS-Dcdc42V12				
10 to 12 hours AEL	46	1	24	29
Hs-GAL4^{M-4};Hs-Drac1N17-104; UAS-Dcdc42V12				
10 to 12 hours AEL	20	1	60	19

*Ages of embryos at time of heat-shock induction of transgenes are shown.

mild DC defective embryos than seen with expression of Drac1N17 alone.

Having seen that both Dcdc42N17 and Dcdc42V12 could cause some rescue of the Drac1N17 DC phenotype, we stained embryos bearing these transgenes to look at the status of the leading edge components. *Hs-GAL4^{M-4};Hs-Drac1N17-104* embryos stained for phosphotyrosine and myosin had complete or nearly complete losses of the leading edge accumulations of these, as seen with previous Drac1N17 inductions (Fig. 6A). *Hs-GAL4^{M-4};Hs-Drac1N17-104;UAS-Dcdc42N17* embryos in the early stages of DC were similarly deficient in leading edge phosphotyrosine and myosin. However, embryos were found in the later stages of DC with reasonable quantities of phosphotyrosine and myosin at the leading edge (Fig. 6B). The presence of such leading edge cytoskeleton is a likely explanation for the partial rescue of Drac1N17-induced DC defects when Drac1N17 is co-expressed with Dcdc42N17 8 to 12 hours AEL.

Staining of *Hs-GAL4^{M-4};Hs-Drac1N17-104;UAS-Dcdc42V12* embryos for phosphotyrosine and DPAK also revealed levels of leading edge components not found following expression of Drac1N17 alone, in fact DPAK levels in excess of controls were found in patches along the leading edge, reminiscent of Dcdc42V12 expression alone (Fig. 6C,D). These results suggest that in the absence of Drac1 signalling, Dcdc42V12 is capable of causing the accumulation of cytoskeletal components and DPAK at the leading edge.

DISCUSSION

A hierarchy of Rho subfamily function is not apparent during DC

We have demonstrated the involvement of four members of the Ras superfamily of small GTPases in the regulation of the cytoskeleton and tissue morphology in DC, an example of epithelial morphogenesis occurring during *Drosophila* embryonic development. These results and our interpretation of them are summarized in Table 2. This work is the most extensive study to date of the participation of all three major classes of Rho subfamily protein in a single developmental process. Comparative studies in cultured mammalian cells have demonstrated a hierarchy of Rho subfamily activity in regulating the actin cytoskeleton and cell morphology (Kozma et al., 1995, 1997; Nobes and Hall, 1995; Allen et al., 1997). In fibroblasts, macrophages, and neuroblastoma cells it appears that Cdc42 can activate the formation of Rac1-mediated lamellipodia. Rac1 in turn can induce the formation of RhoA-dependent focal adhesions and actin stress fibres in fibroblasts, and RhoA-dependent actin cables in macrophages. Thus, it has been proposed that a cascade of Rho subfamily activity in the form: Cdc42→Rac1→RhoA exists (Nobes and Hall, 1995; Allen et al., 1997), although there is evidence for antagonism between Cdc42/Rac1 and RhoA (Lim et al., 1996; Kozma et al., 1997; Allen et al., 1997), and any one of these p21s can be activated directly by extracellular molecules. Our examination of the requirements for Dcdc42, Drac1 and RhoA during DC does not support the existence of such a cascade in regulating the cytoskeleton and morphology of the leading edge epidermal cells. Rather, our results indicate that while all these Rho subfamily proteins are required, they may be functioning largely in parallel.

Dcdc42 is unlikely to be a major activator of Drac1 in our system as Dcdc42N17 has a much milder effect on the leading edge cytoskeleton than Drac1N17, although it is possible that the weaker phenotype of Dcdc42N17 is due to a lower level of transgene expression than Drac1N17. Dcdc42V12 can induce the accumulation of phosphotyrosine nodes and DPAK in the presence of Drac1N17, suggesting that Dcdc42 need not act through Drac1 in its regulation of the leading edge. Finally, Drac1 does not appear to act through RhoA in its regulation of the

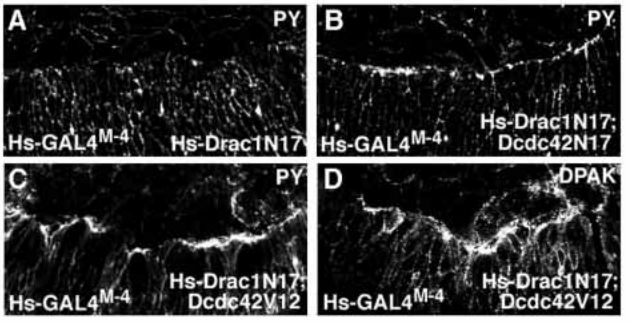

Fig. 6. Both Dcdc42V12 and Dcdc42N17 can partially rescue the leading edge disruption caused by Drac1N7. Embryos were stained with anti-phosphotyrosine antibodies or with anti-DPAK antibodies. (A) *Hs-GAL4^{M-4};Hs-Drac1N17-104* embryo, late in DC, showing loss of phosphotyrosine nodes from leading edge. The same embryo showed loss of leading edge myosin (data not shown) (B) *Hs-GAL4^{M-4};Hs-Drac1N17-104;UAS-Dcdc42N17* embryo, late in DC, showing some phosphotyrosine nodes at the leading edge. The same embryo also showed some accumulation of myosin at the leading edge (data not shown). (C,D) *Hs-GAL4^{M-4};Hs-Drac1N17-104;UAS-Dcdc42V12* embryo double stained for phosphotyrosine (C) and DPAK (D) showing excessive accumulations of both in patches along the leading edge.

Table 2. Proposed roles of small GTPases in DC based on phenotypes caused by expression of mutant transgenes

GTPase	Mutant expressed	Degree of DC	LE phenotype	Proposed role of GTPase
Drac1	Drac1N17	Hole in dorsal surface	Loss of F-actin, myosin, PY, and DPAK	Establishment and/or maintenance of LE cytoskeleton
Dcdc42	Dcdc42N17	Hole or pucker in dorsal surface	Partial loss of F-actin myosin, and PY Loss of DPAK, often with retention of PY Misregulated contraction	Establishment and/or maintenance of LE cytoskeleton Control of LE DPAK levels; DPAK may downregulate LE cytoskeleton
	Dcdc42V12	Dorsal surface closed but puckered	Elevated DPAK Some losses of F-actin myosin, PY, DPAK	
RhoA	DRhoAN19	Hole or pucker in dorsal surface	Disruption of LE cytoskeleton and loss of contraction in segment border cells	Regulation of LE cytoskeleton in segment border cells
	RhoAV14	Dorsal surface closed but puckered	Distribution of LE components normal	
Ras1	Ras1N17	Dorsal surface closed but puckered	Partial loss of F-actin myosin and PY	Establishment and/or maintenance of LE cytoskeleton Control of LE DPAK levels
	Ras1Q13	Hole or pucker in dorsal surface	Elevated DPAK	

LE, leading edge. PY, phosphotyrosine.

leading edge cytoskeleton in the cells outside the segment border regions, as DRhoAN19 expression has no effect on these cells.

The leading edge cytoskeleton is established and/or maintained by Drac1 and Dcdc42

Of the p21s tested here, Drac1is likely to be a key player in the establishment and/or maintenance of the leading edge cytoskeleton as it can cause severe disruption of this structure. This regulation of the leading edge cytoskeleton by Drac1 is apparently largely carried out by a Jun amino-terminal kinase (JNK) cascade required for DC, as the leading edge cytoskeleton is lost in Djun mutant embryos, and constitutively active Djun can significantly rescue the DC phenotype caused by Drac1N17 (Hou et al., 1997). Furthermore, Drac1V12 can induce ectopic expression of *dpp* and *puc*, two JNK cascade target genes (Glise and Noselli, 1997).

Dcdc42N17 can cause partial losses of the leading edge cytoskeleton, and Dcdc42V12 can induce the accumulation of leading edge components in the presence of Drac1N17. These results suggest that Dcdc42 can contribute to the formation of the leading edge cytoskeleton. Dcdc42V12, like RacV12, may be acting through the JNK cascade in this regulation of the leading edge cytoskeleton, as Dcdc42V12 can induce ectopic expression of *dpp* and *puc* (Glise and Noselli, 1997). However, Dcdc42 is less effective than DracV12 at causing these ectopic inductions in the dorsal epidermis. That Dcdc42N17 has a significantly weaker effect on the leading edge cytoskeleton than Drac1N17 may be because Cdc42 is a less effective inducer of the JNK cascade in the leading edge.

Dcdc42 may down regulate the leading edge cytoskeleton through DPAK

We have demonstrated that ectopic expression of Dcdc42 mutants can have striking effects on the levels of DPAK at the leading edge, with Dcdc42N17 causing loss and Dcdc42V12 causing premature accumulation. This control of DPAK localization at the leading edge may be analogous to the Cdc42/Rac1-mediated association of α-PAK with peripheral actin structures and focal complexes in cultured mammalian cells (Manser et al., 1997; Dharmawardhane et al., 1997), as the leading edge represents a peripheral accumulation of both F-actin and focal complex-like phosphotyrosine-rich structures (Harden et al., 1996). We previously noted that segment border cells showed transient loss of the leading edge cytoskeleton, and that this loss was preceded by elevated DPAK levels in these cells (Harden et al., 1996). We speculated that elevated DPAK may lead to the down regulation of the cytoskeleton in these cells. PAK's role in dissolution of the actin cytoskeleton has been shown in HeLa cells, where transfection of constitutively active PAK causes breakdown of actin stress fibres and loss of focal complexes (Manser et al., 1997). We have now demonstrated that Dcdc42V12 can increase DPAK levels at the leading edge during DC to levels normally seen only at the end of DC. Dcdc42V12 can also cause loss of the leading edge cytoskeleton (including the phosphotyrosine-rich structures) and leading edge DPAK in some embryos. These two seemingly opposite effects of Dcdc42V12 expression are not confined to separate developmental stages, as they can occur together in a single embryo (Fig. 3C). It is possible that DPAK, activated by Dcdc42, is localizing with the leading edge cytoskeleton and phosphotyrosine-rich complexes and contributing to their down regulation. This breakdown is first executed transiently in the segment border cells and then permanently all along the leading edge at the end of DC. Following DC the former leading edge shows no elevation of F-actin, myosin, phosphotyrosine, or DPAK. Dcdc42V12 may be capable of elevating DPAK sufficiently along the leading edge to cause premature down regulation of the cytoskeleton,

as seen in about 10% of Dcdc42V12-expressing embryos. When the cytoskeleton is lost from the leading edge, the leading edge DPAK may be lost with it, if DPAK localization at the leading edge is dependent on interaction with the cytoskeleton. In an embryo such as that shown in Fig. 4C, the patches deficient in leading edge DPAK and F-actin may represent regions that previously had high levels of DPAK. These high levels of DPAK could then have caused down regulation of the cytoskeleton, which in turn could have resulted in loss of DPAK from the leading edge.

Expression of Dcdc42N17 by a weak heat-shock driver causes a pronounced bunching together of the segments at the leading edge. This may be due to loss of the transient down regulation of the leading edge cytoskeleton that normally occurs at the segment borders, resulting in a misregulated contraction of the leading edge. Our model for DPAK function during DC remains speculative in the absence of data on the effects of disrupting DPAK activity during DC, but is is consistent with the known effects of PAK on the cytoskeleton in mammalian cells.

Dcdc42 may have two conflicting roles during DC: establishment and/or maintenance of the leading edge cytoskeleton versus breakdown of the leading edge cytoskeleton through DPAK. The varying phenotypic effects seen with Dcdc42 transgene expression by different drivers may reflect changes in the balance of these two roles. The likelihood of two opposing functions for Dcdc42 is strengthened by the results of co-expressing Dcdc42N17 with Drac1N17, where Dcdc42N17 shows a temporal progression from worsening to partially rescuing Drac1N17-induced DC defects. We interpret these results as indicating that during DC, Dcdc42 shifts from positively to negatively regulating the leading edge cytoskeleton. Late in DC, Dcdc42N17 may be able to ameliorate the deleterious effects of Drac1N17 on the leading edge cytoskeleton, through reduction of leading edge DPAK levels.

RhoA is required for the integrity of the leading edge cytoskeleton in cells flanking the segment borders

We have demonstrated that expression of either dominant negative or constitutively active mammalian RhoA disrupts DC. Expression of a dominant negative *Drosophila RhoA* transgene has the same effect, and embryos transheterozygous for null alleles of the *RhoA* gene exhibit defects in the dorsal epidermis (Strutt et al., 1997). Dominant negative *Drosophila RhoA* expression leads to loss of leading edge components and a loss of anterior-posterior contraction in several cells flanking each segment border. Such losses are also seen in the segment border cells of wild-type embryos, but they occur at only a few segment borders of an embryo at a given time, and appear to be transient (Harden et al., 1996). In *Hs-GAL4^{M-4};UAS-DRhoAN19* embryos the leading edge losses are present at every segment border of each embryo examined and appear to persist, leading to uneven contraction of the leading edge.

One possible explanation for the *Hs-GAL4^{M-4};UAS-DRhoAN19* phenotype is that RhoA is required for the assembly of the leading edge cytoskeleton in the segment border cells, perhaps downstream of Drac1. An alternative interpretation is that RhoA is functioning as a negative regulator of the leading edge cytoskeletal losses that occur in wild-type embryos, and that when RhoA function is absent these losses become permanent instead of transient. We have speculated that Dcdc42, acting through DPAK, may be regulating the leading edge cytoskeleton in the segment border cells. Antagonism between RhoA and Cdc42 has been noted in some studies in cultured mammalian cells (Lim et al., 1996; Kozma et al., 1997; Allen et al., 1997), and such antagonism is one possible mechanism for RhoA function in the segment border cells. RhoA function in the segment border cells could be examined further by co-expressing RhoA transgenes with Drac1 or Dcdc42 transgenes and examining the net phenotypic effects.

Ras1 may be acting through Dcdc42 and/or Drac1 in regulating DC

Expression of Ras1N17 caused partial losses of the leading edge cytoskeleton, and Ras1Q13 increased DPAK levels at the leading edge. Thus, Ras1 has phenotypic effects similar to those of Dcdc42 and Drac1. There is mounting evidence that the Rho subfamily proteins lie downstream of Ras, contributing to its ability to cause transformation and regulate the actin cytoskeleton (Symons, 1996; Qiu et al., 1997). Our results are consistent with Ras1 activating Dcdc42 and/or Drac1 during DC, although given that Ras1 expression has milder phenotypic effects than Dcdc42 or Drac1, we would not propose it as a chief activator of either of these p21s. Our finding that Ras1Q13 can increase DPAK levels at the leading edge is the first demonstration of Ras having an effect on the behaviour of a PAK family member. Interestingly, it has recently been shown that kinase deficient PAK1 mutants can inhibit Ras transformation, indicating that PAK may be a component of Ras signalling (Tang et al., 1997, 1998). Although Ras1Q13, like Dcdc42V12, elevated DPAK levels at the leading edge it did not cause the losses of leading edge components seen following Dcdc42V12 expression. Looking at these results in the context of our model for DPAK function, it may be that RasQ13 does not increase DPAK accumulation at the leading edge to a level sufficient to cause down regulation of the cytoskeleton.

Dorsal closure as a system for dissecting the regulation of epithelial morphogenesis

Recently, DC has emerged as a very promising system for the study of epithelial morphogenesis (reviewed by Knust, 1997; Martin-Blanco, 1997; Noselli, 1998). Analysis of mutants in DC has revealed molecules that can be placed in three categories. The first group consists of proteins directly associated with the cytoskeleton, the second group members of a JNK signalling cascade, the third members of a Decapentaplegic (Dpp) signalling cascade. Links between the JNK and Dpp cascades have been demonstrated, but how these cascades regulate the cytoskeleton to actually cause morphogenesis is unknown (Glise and Noselli, 1997; Hou et al., 1997; Riesgo-Escovar and Hafen, 1997). The Rho subfamily p21s are likely candidates for a link between signalling to the nucleus and cytoskeletal change. In mammalian cells, Rac and Cdc42 participate in JNK cascades which presumably lead to transcriptional regulation. Rac and Cdc42 are also capable of inducing rapid changes in the cytoskeleton, even when their ability to activate JNK is impaired (reviewed by Van Aelst and D'Souza-Schorey, 1997).

As discussed above, Drac1 and Dcdc42 also appear to be components of a JNK cascade in DC and thus may be controlling the transcriptional upregulation of various gene products, but they may also be acting directly on the leading edge cytoskeleton. Myosin protein levels and DPAK transcript levels are higher in the leading edge cells than elsewhere in the lateral epidermis (Young et al., 1993; Harden et al., 1996) and these increases may be mediated by the JNK cascade. Once such proteins are manufactured in the leading edge cells, they must then be assembled into the cytoskeletal structures that drive morphogenesis. It is at this point that the Rho subfamily may again be required. There is a growing family of molecules that are likely to be direct cytoskeletal effectors for the Rho subfamily and it will be of interest to characterize such molecules in *Drosophila* and evaluate their roles in DC.

Our study has demonstrated that at least four different small GTPases are required for DC. Further study of the involvement of these molecules in DC should help unravel the interactions between the many proteins participating in this process and provide insight into how epithelial morphogenesis is controlled.

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