A dominant inhibitory version of the small GTP-binding protein Rac disrupts cytoskeletal structures and inhibits developmental cell shape changes in *Drosophila*

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SUMMARY

The Rho subfamily of Ras-related small GTP-binding proteins is involved in regulation of the cytoskeleton. The cytoskeletal changes induced by two members of this subfamily, Rho and Rac, in response to growth factor stimulation, have dramatic effects on cell morphology. We are interested in using *Drosophila* as a system for studying how such effects participate in development. We have identified two *Drosophila* genes, *DRacA* and *DRacB*, encoding proteins with homology to mammalian Rac1 and Rac2. We have made transgenic flies bearing dominant inhibitory (*N17DRacA*), and wild-type versions of the *DRacA* cDNA under control of an *Hsp70* promoter. Expression of the *N17DRacA* transgene during embryonic development causes a high frequency of defects in dorsal closure which are due to disruption of cell shape changes in the lateral epidermis. Embryonic expression of *N17DRacA* also affects germband retraction and head involution. The epidermal cell shape defects caused by expression of *N17DRacA* are accompanied by disruption of a localized accumulation of actin and myosin thought to be driving epidermal cell shape change. Thus the Rho subfamily may be generating localized changes in the cytoskeleton during *Drosophila* development in a similar fashion to that seen in mammalian and yeast cells. The Rho subfamily is likely to be participating in a wide range of developmental processes in *Drosophila* through its regulation of the cytoskeleton.

Key words: *Drosophila*, Rac, small GTP-binding protein, dorsal closure, cell shape, cytoskeleton

INTRODUCTION

The participation of the small guanosine triphosphate (GTP)-binding protein Ras in numerous signal transduction pathways has been extensively investigated. Ras cycles between an active GTP-bound and an inactive GDP (guanosine diphosphate)-bound state. This cycling is regulated by a number of proteins including guanine nucleotide exchange factors, GDP dissociation inhibitors, and GTPase-activating proteins (GAPs). These proteins, along with others not directly interacting with Ras, have been identified in both biochemical and genetic studies and are being placed into specific pathways extending from the cell surface to the nucleus. One of the best characterized of these pathways is the sevenless signalling cascade of *Drosophila* photoreceptor development (Simon et al., 1993), indeed a major contribution to our present understanding of Ras signalling has come from studies in *Drosophila*. Although most of this work is based on mutants isolated by genetic approaches, it has been demonstrated that activated forms of *Drosophila* Ras genes created by site-directed mutagenesis can produce developmental abnormalities when put into flies bearing wild-type Ras genes (Bishop and Corces, 1988; Fortini et al., 1992; Brand and Perrimon, 1993; Lu et al., 1993). Additionally, injection of a dominant inhibitory mutant form of mammalian Ras1 protein into wild-type embryos has a phenotypic effect (Lu et al., 1993).

An increasing amount of attention has been paid to the other small GTP-binding proteins (p21s), related to Ras, which also appear to be involved in a wide range of processes. Biochemically, these proteins show many similarities to Ras, and much of what is known about Ras has been applied to their study. *Drosophila* is a suitable system for the study of these p21s, given the significant contribution of the fly to the understanding of Ras. As with Ras, it should be possible to characterize genetically newly isolated genes encoding *Drosophila* p21s by expressing the activated or dominant inhibitory mutant proteins in transgenic flies and looking for phenotypes. Such phenotypic effects can also provide a starting point for a developmental analysis of p21 function. The ease with which the *Drosophila* embryo in particular, can be examined and manipulated should make it possible to determine how p21-induced changes at the cellular level are integrated into the development and function of a multicellular eukaryote.

We are interested in characterizing the Rho subfamily of...
Ras-related p21s. Changes in cell shape during growth and differentiation are exquisitely influenced by these molecules, which affect the morphology of cells by regulating the response of the cytoskeleton to external factors and its participation in cell division. The Rho subfamily comprises the mammalian RhoA, RhoB, RhoC, Rac1, Rac2, TC10 and Cdc42Hs proteins, and the Saccharomyces cerevisiae proteins, Cdc42Sc, Rho1 and Rho2 (Vincent et al., 1992). The microinjection of a constitutively active form of RhoA into Swiss 3T3 fibroblasts leads to the assembly of focal adhesions and actin stress fibers, two events in the normal cellular response to growth factors (Ridley and Hall, 1992). Rac1 participates in the process of membrane ruffling, which is another growth factor-induced rearrangement of actin filaments (Ridley et al., 1992). The Cdc42Sc protein is involved in bud formation, apparently by interaction with the actin network (Adams et al., 1990, Johnson and Pringle, 1990).

We began our study by looking for Drosophila homologues of the Rac proteins. Two highly homologous Rac (Ras-related C3 botulinum toxin substrate; Didsbury et al., 1989) proteins, termed Rac1 and Rac2, have been identified in mammals (Didsbury et al., 1989, Shirsat et al., 1990, Moll et al., 1991). Rac2 is specific to myeloid cells whereas Rac1 is expressed in a wider range of tissues, including myeloid cells. Rac2 is an activator of superoxide production in phagocytes (Knaus et al., 1991). Rac1 can also carry out this function in addition to its role as a cytoskeletal regulator (Abo et al., 1991). A single C. elegans Rac with greater homology to mammalian Rac1 than Rac2 has been identified, although there is evidence for a second Rac protein in this species (Chen et al., 1993).

We report here the cloning and characterization of two Drosophila genes, DRAcA and DRAcB, encoding proteins highly homologous to mammalian Rac1 and Rac2. We have expressed wild-type and dominant inhibitory versions of the DRAcA cDNA under control of a heat shock promoter. Induction of the dominant inhibitory DRAcA transgene during embryonic development causes defects in dorsal closure, apparently by hindering the epidermal cell shape changes normally associated with this process. We show that an actin/myosin structure believed to be driving these cell shape changes (Young et al., 1993) is disrupted by expression of the dominant inhibitory DRAcA transgene.

**MATERIALS AND METHODS**

**DNA and RNA analysis**

Preparation, blotting and hybridization of DNA and RNA were performed using standard procedures (Sambrook et al., 1989). For the assessment of heat shock induction of the DRAcA transgenes, adult females between 1 and 7 days old were placed in vials and either maintained at 22°C or heat shocked for 1 hour at 37°C. Following heat shock, flies were immediately frozen and total RNA prepared from them and their control siblings as described by Chia et al. (1985).

**Low-stringency library screening**

A human Rac1 cDNA was labelled by random priming and used to screen a Drosophila genomic library (Stratagene) in the λ FIX II vector. Filters were prehybridized and hybridized in a buffer containing 35% formamide, 5× SSC, 5× Denhardt’s, 0.1 M sodium phosphate (pH 6.8) and 100 μg/ml denatured salmon sperm DNA. Filters were prehybridized for 4 hours at 42°C, the probe added, and then hybridized for 16 hours at 42°C. Filters were washed in 2× SSC, 0.1% SDS at room temperature for 20 minutes, and then in 2× SSC, 0.1% SDS at 55°C for 30 minutes. Adult and 2-14 hour embryo cDNA libraries (Stratagene) in λ ZAP vectors were screened with a subclone from a genomic positive under the same low stringency conditions.

**DNA sequencing**

Genomic DNA and cDNA restriction fragments were subcloned into Bluescript vectors (Stratagene) and sequenced by the dyeoxy chain-termination method using Sequenase (United States Biochemical). cDNAs were sequenced on both strands, and specific 17-mer oligonucleotide primers were used to complete sequences.

**In situ hybridization to polytene chromosomes**

Polytene chromosomes were prepared from wild-type (Canton S) or transgenic third instar larvae and hybridized with biotinylated DNA probes, as described by Ashburner (1989), but with the acetylation step omitted. Peroxidase detection of signals was done with a Deteck-1-HRP kit (Enzo Biochemicals).

**Whole-mount in situ hybridization to embryos**

Wild-type (Canton S) embryos were hybridized with digoxigenin-labelled DNA probes as described by Ashburner (1989), using the Boehringer Mannheim non-radioactive DNA labelling and detection kit. Embryos were staged according to Campos-Ortega and Hartenstein (1985).

**Oligonucleotide-directed mutagenesis and heat shock constructs**

Single base pair changes were introduced into DRAcA cDNAs using the Transformer site-directed mutagenesis kit (Clontech). The activated DRAcA mutation was created using the oligonucleotide GTGGGCGACGTGGCGTG, with the base change converting amino acid 12 from Gly to Val (underlined). The N17DRAcA mutation was created using the oligonucleotide GTGGAAAGAAGCTGGCGTGCTG, with the base change converting amino acid 17 from Thr to Asn (underlined). EcoRI/XhoI restriction fragments containing the entire coding regions of the mutated and wild-type DRAcA cDNAs were each subcloned into the pcCaSpeR vector (Pirrotta, 1988). pcCaSpeR carries a white ‘mini-gene’ as a marker and an Hsp70 promoter driving the expression of the inserted sequence. The mutated sites in the activated DRAcA and N17DRAcA pcCaSpeR constructs were confirmed by direct sequencing of the pcCaSpeR DNA using an oligonucleotide primer.

**Germ-line transformation**

Injection of the DRAcA pcCaSpeR constructs into Df(1)yw embryos was performed as described by O’Connor and Chia (1993). Injected embryos were cultured at 22°C, and surviving adults were individu- maily mated to Df(1)yw flies. Progeny with a pcCaSpeR insertion were identified by the presence of varying degrees of w+ eye color. Balanced lines were established for each insertion event, and the number and position of inserts determined by polytene chromosome in situ hybridization. Heat shocks were performed by placing embryos in vials containing yeast-glucose medium and immersing them in a Grant LTD 6 water bath set at 37°C. For some of the heat shocks, individuals were directly immersed in pre-warmed distilled water. For each heat shock experiment, a minimum of 100 embryos were examined for phenotypic effects.

**Cuticle preparations**

Embryos were cleared by mounting in Hoyer’s medium as described by Ashburner (1989), except that embryos were not fixed before mounting.

**Immunocytochemistry**

Antibody staining of embryos was done using standard techniques
(Ashburner, 1989). Peroxidase-conjugated goat secondary antibodies (Jackson Immunoresearch Laboratories) were detected using the glucose oxidase-DAB-nickel method of Hsu et al. (1988). Fluorescent detection of antibodies was performed using biotinylated secondary antibodies (Vector Laboratories), and TRITC-labelled or FITC-labelled streptavidin (Vector Laboratories). Fixed embryos, for staining of filamentous actin, which had their vitelline membranes removed in 80% ethanol, were incubated for 25 minutes in 0.5 µg/ml TRITC-labelled phallolidin (Sigma) in PBS, and washed for 20 minutes in several changes of PBS. All fluorescent-labelled embryos were mounted in 90% glycerol with 0.5 mg/ml p-phenylenediamine (Sigma) as an anti-bleaching agent, and examined by scanning laser confocal microscopy using a Biorad MRC 600 microscope. Confocal images were prepared for publication using Adobe Photoshop (Adobe Systems).

RESULTS

Molecular cloning of genes encoding Drosophila Rac proteins

A Drosophila genomic library was screened with a human Rac1 cDNA (Didsbury et al., 1989) under low-stringency conditions. Twenty-six hybridizing phages were isolated, and one of the strongest positives was selected for further analysis. A 0.9 kb PstI restriction fragment that strongly hybridized to the human Rac1 cDNA was identified in the genomic phage. This fragment, when sequenced, contained an open reading frame (ORF) encoding a protein highly homologous to human Rac1 and Rac2. The 0.9 kb restriction fragment was used to screen Drosophila embryonic and adult cDNA libraries under low stringency conditions. Both libraries yielded cDNAs encoding two different Rac1-homologous proteins. One group of cDNAs, derived from a gene we termed DRacA, contained an ORF identical to that within the original genomic phage. When polytene chromosome in situ hybridizations were performed with one of the DRacA cDNAs and the genomic phage, both clones hybridized to cytological position 61F5 on chromosome 3L (data not shown). A representative from the other group of cDNAs, derived from the DRacB gene, was found to colocalize with another phage isolated in the genomic screen at cytological position 66A1 on chromosome 3L (data not shown). Therefore, we have identified two genes at different cytological locations encoding proteins with strong homology to the human Rac proteins.

Nucleotide sequence of the DRacA and DRacB cDNAs

The nucleotide sequences of the DRacA and DRacB cDNAs, along with their predicted protein sequences, are given in Fig. 1. Both nucleotide sequences are composites from data on several cDNA clones. The 3’ end of the DRacB sequence has not been determined as all DRacB cDNAs obtained terminated upstream of the poly(A) tail at an A-rich stretch in the 3’ UTR. The presence of this A-rich region has been confirmed by genomic sequencing (data not shown). The predicted amino acid sequences of the DRacA and DRacB proteins are aligned with the human Rac1 and Rac2 sequences in Fig. 2. The alignments of the predicted Drosophila Rac proteins with Rho subfamily proteins other than Rac give significantly lower homologies (data not shown). The DRacA cDNA encodes a product with 91.7% (176/192) and 89.1% (171/192) identities to the human Rac1 and Rac2 proteins (Didsbury et al., 1989), respectively. The percentage identities of the DRacB encoded protein with Rac1 and Rac2 are 89.6% (172/192) and 88.0% (169/192), respectively. The two Drosophila proteins are 93.2% (179/192) identical, while the human Rac proteins are 92.2% (177/192) identical. The only domain demonstrating major differences among the four proteins is towards the carboxyl terminus (Fig. 2). This region varies considerably in the Ras family, even within the subgroups. Although both Drosophila proteins are marginally more like Rac1 than Rac2, the DNA sequences encoding them are more homologous to the Rac2 cDNA sequence than that of Rac1. The DRacA coding sequence has 74.1% (429/579) and 81.7% (473/579) identity with the coding sequences of Rac1 and Rac2, respectively. The coding sequence of DRacB compared with those of Rac1 and Rac2, has identities of 72.4% (419/579) and 77.2% (447/579), respectively. These data do not readily allow the relationship between the two Drosophila Rac genes and the genes encoding Rac1 and Rac2 to be determined, and we have chosen to assign new names to these Drosophila genes.

Developmental profile of DRacA and DRacB transcription

The DRacA and DRacB genes are expressed throughout development, without any significant fluctuations in transcript levels (Fig. 3). The estimated length of the DRacA transcript, 2.0 kb, is in fairly good agreement with the cDNA length of 1.8 kb. The DRacB probe detects transcripts of 2.0 and 2.9 kb; the 2.0 kb transcript may be that of DRacA being detected by cross homology. The large discrepancy between these transcript lengths and the 0.9 kb cDNA sequence can be explained by the truncation of the 3’ UTR sequences of the DRacB cDNA (see above). The 2.9 kb transcript detected by the DRacB cDNA is expressed at higher levels in the adult body than the head. We have not been able to detect any embryonic tissue specificity of expression of the DRacA and DRacB genes, which appear to be expressed ubiquitously during embryogenesis (data not shown).

Creation of DRacA mutant transgenes under Hsp70 control

Dominant mutations of Ras family genes, useful for genetic analysis, can be created by site-directed mutagenesis. A mutant p21 may then be introduced into flies under control of a heatshock promoter. A dominant mutation of Rac is the inhibitory form by changing amino acid 12 from Gly to Val, analogous to the V12 mutation in oncogenic Ras. Activated Rac has reduced intrinsic GTPase activity and does not respond to GTPase activating proteins (Diekmann et al., 1991). The activated DRacA cDNA was placed under control of the Hsp70 promoter, along with the transgenic vector and inserted into two different strains of flies. Both strains were then raised at 25°C under low-stringency conditions. Twenty-six hybridizing phages were isolated, and one of the strongest positives was selected for further analysis. A 0.9 kb PstI restriction fragment that strongly hybridized to the human Rac1 cDNA (Didsbury et al., 1989) under low-stringency conditions. The estimated length of the DRacA transcript, 2.0 kb, is in fairly good agreement with the cDNA length of 1.8 kb. The DRacB probe detects transcripts of 2.0 and 2.9 kb; the 2.0 kb transcript may be that of DRacA being detected by cross homology. The large discrepancy between these transcript lengths and the 0.9 kb cDNA sequence can be explained by the truncation of the 3’ UTR sequences of the DRacB cDNA (see above). The 2.9 kb transcript detected by the DRacB cDNA is expressed at higher levels in the adult body than the head. We have not been able to detect any embryonic tissue specificity of expression of the DRacA and DRacB genes, which appear to be expressed ubiquitously during embryogenesis (data not shown).
amino acid 17 from Thr to Asn, a Rac protein, N17Rac, is produced with a preferential affinity for GDP, and is consequently likely to be locked in an inactive state (Ridley and Hall, 1992). Dominant inhibitory Rac and Ras proteins probably inhibit their normal counterparts by sequestering guanine nucleotide exchange factors whose function is the reactivation of GDP-bound p21s (Ridley and Hall, 1992; Farnsworth and Feig, 1991). That a dominant inhibitory p21 can exert an effect at the level of the whole fly has been demonstrated for Ras1 in *Drosophila* (Lu et al., 1993). A dominant inhibitory DRacA transgene (*N17DRacA*) under *Hsp70* control was constructed and injected into embryos. Four insertion lines were established for the *N17DRacA* transgene, with two each on the second (N17-5, N17-122) and third (N17-104, N17-119) chromosomes. The second chromosome insertions were balanced over a CyO chromosome, and those on the third over a TM3 chromosome. The N17-5 and N17-104 lines are homozygous viable, the N17-122 line homozygous semilethal, and the N17-119 line homozygous lethal. In all four lines heterozygous flies appear normal when maintained at 22°C. To confirm that the transgenes were inducible by heat shock, adult flies were incubated for 1 hour at 37°C and then northern analysis was carried out (Fig. 4). There is a high level of heat shock induced *N17DRacA* expression for all four transgenes. There is an additional, inducible transcript in the N17-119 and N17-122 lines. We do not know how this transcript is produced, but an unexplained extra transcript has also been noted for heat shock expression of activated *DRas2* (Bishop and Corces, 1988).

As a control for the *N17DRacA* studies, and to study the effects of over expression of a Rac gene, we have injected embryos with a wild-type *DRacA* transgene under *Hsp70* control. We have obtained an X chromosome insertion of this construct, WT-10, which is homozygous viable. The level of induction of the wild-type RacA transgene of heat shocked and control adult flies is similar to that seen for *N17DRacA* (data not shown).

**Heat shock expression of two copies of a dominant inhibitory DRacA transgene during embryogenesis disrupts dorsal closure**

To study the effects of transgene expression on embryogenesis, we performed heat shocks on embryos bearing wild-type and *N17DRacA* transgenes, left them to develop at 25°C, and

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**Fig. 1. Nucleotide sequence and conceptual translation of *Drosophila* Rac cDNAs.**

(A) cDNA sequence of *DRacA*, with consensus polyadenylation signals (AATAAA) underlined.

(B) cDNA sequence of *DRacB*, which is truncated at the 3′ end (see text).
examined their cuticles. Embryos were aged at 25°C before shifting to 37°C. Heat shock causes high levels of lethality in wild-type embryos during early embryogenesis, due to the inability of embryos to synthesize heat shock proteins prior to the cellular blastoderm stage (Walter et al., 1990). Thus, we have not examined the effects of transgene induction prior to 4 hours after egg laying (AEL). Initial heat shocks of 1 hour were performed on embryos aged from 4-7 hours AEL. Many of the embryos from the N17-5 and N17-104 lines, bearing two copies each of *N17DRacA*, exhibited defects in the dorsal surface and lower numbers of individuals were found with failures of germband retraction. No obvious defects were found in any of the embryos from the WT-10 strain in which females carry two copies of the wild-type *DRacA* transgene. A more detailed analysis of the effects of transgene induction during embryogenesis was done on the homozygous N17-104 line and the WT-10 line.

N17-104 embryos were collected for 1 hour and aged for 4-12 hours prior to a 30 minute heat shock. We also examined the effects of inducing *N17DRacA* rapidly for a short period of time, by immersing embryos directly in prewarmed distilled water for 10 minutes. Both procedures gave similar effects. Defects in the dorsal surface were seen in at least 35% of heat shock embryos shocked at intervals from 4-5 hours to 9-10 hours AEL (Fig. 5A-E). A few individuals were also seen with dorsally open heads (Fig. 5E) or failures in germband retraction (Fig. 5F). Embryos heat shocked at 11-12 hours or 12-13 hours AEL did not exhibit any cuticle defects. The highest number of defects was seen in embryos heat shocked at 6-7 hours AEL. Of the cuticles from this interval, 55% exhibited obvious abnormalities in the dorsal surface. Many had a small ‘scab’ (Fig. 5A), while others had 1 or several holes in the dorsal cuticle (Fig. 5B-D). The cuticle around all these abnormalities was distorted into a series of folds. The most extreme example of a dorsal defect was a missing dorsal surface (Fig. 5E). Inductions of 1 hour applied to younger embryos produced a dramatic increase in the frequency of germband retraction failures and missing dorsal surfaces. For example, doubling the length of the heat shock applied to embryos aged 5-6 hours AEL resulted in a ten-fold increase in the numbers of defective individuals. One hour heat shocks on WT-10 embryos collected and aged in the same manner as the N17-104 embryos produced no apparent cuticle abnormalities.

A hole or scab in the dorsal cuticle is indicative of a defect in dorsal closure, a process probably dependent on cell shape changes (see review by Martinez Arias, 1993). During dorsal closure of the epidermis over the amnioserosa, the shape of epidermal cells in the dorsal region changes dramatically. Beginning with cells immediately flanking the amnioserosa, there is an elongation along the dorsoventral axis that gradually ‘spreads’ ventrally through the epidermis. These cell shape changes stretch the apposing sides of the lateral epidermis until they meet along the dorsal midline (Young et al., 1993). During dorsal closure, the heavy chain of nonmuscle myosin is found at a high level along what has been termed the leading edge, which is composed of the dorsal edges of those epidermal cells immediately adjacent to the amnioserosa (Young et al., 1993). The nonmuscle myosin probably contributes to the elongation of these cells by participating with actin in forming a dorsal constriction (Young et al., 1993). Shape changes in more ventrally located cells could then occur as a resultant, passive response. Mutations in the zipper (*zip*) locus (Nüsslein-Volhard et al., 1984), which encodes the nonmuscle myosin heavy chain (Young et al., 1993), cause a defect in the dorsal cuticle similar to the dorsal holes produced by expression of *N17DRacA*. Hereafter we will use the terms ‘nonmuscle myosin heavy chain’ and ‘myosin’ interchangeably.

Since members of the Rho subfamily are known to regulate the cytoskeleton, we reasoned that expression of *N17DRacA* may interfere with the cytoskeletal changes required for the epidermal cell elongation associated with dorsal closure. To look at epidermal cell shape changes, we did further heat shocks on N17-104, WT-10 and wild-type embryos, allowing them to develop at 25°C, and stained them with antibodies against Fasciclin III (Patel et al., 1987). Fasciclin III is a glycoprotein expressed on all epidermal cell surfaces except the dorsal ends of the cells flanking the amnioserosa (Fig. 6B). When N17-104 embryos aged 5-7 hours AEL were given 10 minute, 30 minute or 1 hour heat shocks, many individuals were found with incomplete dorsal closure. Examination of the lateral epidermis of such individuals revealed that cells were disorganized, not elongated properly, and that some cells flanking the dorsal hole showed an abnormal staining for Fasciclin III on their dorsal sides (Fig. 6E-H). Occasionally, dorsal closure occurred in patches (Fig. 6H), producing a multiple dorsal hole phenotype as described earlier for the cuticle preparations. Thus, expression of *N17DRacA* disrupts the epidermal cell shape changes that normally occur during dorsal closure. The control heat shocked WT-10 and wild-type embryos were indistinguishable from wild-type embryos maintained at 25°C (data not shown).

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**Fig. 2.** Alignment of the predicted amino acid sequences of the *Drosophila* Rac proteins DRacA and DRacB with human Rac1 (HsRac1) and human Rac2 (HsRac2) (Didsbury et al., 1989).
Expression of dominant inhibitory DRacA affects the distribution of α-spectrin, a cytoskeletal protein

Fasciclin III is not expressed in the cells of the amnioserosa, and staining for this protein does not allow one to determine easily the fate of these cells in N17-104 embryos with defective dorsal closure. The hindgut does not appear to be covered by any layer of cells in late embryos with a dorsal hole, such as that in Fig. 6E. Thus, the amnioserosa cells may disappear into the interior of the embryo, as is believed to occur normally (Martinez Arias, 1993). To confirm this, we collected N17-104, wild-type and WT-10 embryos and heat shocked them for 30 minutes or 1 hour at 4-7 hours AEL. Embryos were then allowed to develop before staining with antibodies against α-spectrin (Pesacreta et al., 1989), a membrane skeleton protein that clearly defines the cells of the amnioserosa and epidermis. Fig. 7 shows the dorsal opening of heat shocked wild-type (A) and N17-104 (B) embryos late in dorsal closure. The wild-type embryo shows strong staining in the amnioserosa, as well as along the leading edge. The heat shocked embryo shows greatly reduced staining in the amnioserosa and no visible staining of the leading edge. The cell shape changes in this embryo have not been as dramatically affected as those of the individuals shown in Fig. 6E-H, although clearly abnormal cells are seen. The shapes of the amnioserosa cells are normal. Induction of the WT-10 transgene had no effect on α-spectrin staining (data not shown). In those N17-104 embryos that do not complete dorsal closure, the amnioserosa cells disappear into the interior of the embryo, but occasionally leave clusters of weakly stained cells at the surface (data not shown). Further study is required to determine if the fate of the amnioserosa cells is affected by N17DRacA expression. The α-spectrin staining patterns of N17-104, WT-10 and wild-type embryos maintained at 25°C were all indistinguishable from that shown in Fig. 7A (data not shown).

The loss of α-spectrin staining along the leading edge suggested that induction of N17DRacA was affecting the cytoskeleton along the dorsal sides of the epidermal cells flanking the amnioserosa. A plausible explanation for the poor cell shape changes in the epidermis following N17DRacA induction is that the dorsally localized actin/myosin structure in the cells of the leading edge is disrupted. To test this, we looked at the distribution of actin and myosin in embryos following transgene induction.

Expression of dominant inhibitory DRacA disrupts accumulation of actin and myosin along the leading edge

N17-104, WT-10 and wild-type embryos were heat shocked for 30 minutes or 1 hour at 4-7 hours AEL and aged until a point when most of the embryos were in the process of dorsal closure, i.e. late stage 14/early stage 15. Embryos were fluorescently stained for filamentous actin using phalloidin, or for nonmuscle myosin heavy chain using antibodies (Kiehart and Feghali, 1986), or both. Young et al. (1993) have described an accumulation of actin and myosin along the leading edge, as revealed by antibody staining. Our results confirm this finding, and show that the actin along the leading edge is filamentous (Fig. 8A,B). Following induction of N17DRacA, the accumulation of actin and myosin along the leading edge is affected to varying degrees. There is a good correlation between the severity of the heat shock effect, as gauged by the size of the dorsal opening relative to the developmental stage and the epidermal cell shapes, and the loss of

![Fig. 3. Developmental profile of transcription of the DRacA and DRacB genes. The lanes contain about 2 µg poly(A)+ RNA from embryonic stages (0-6 hours, 6-12 hours, and 12-24 hours AEL), larval stages (L1, first instar; L2, second instar; L3, third instar), 3 to 4 day old pupae (P3-4), adult head (Head) and adult body (Body). The top panel shows the blot probed with a DRacA cDNA, which detects a transcript of 2.0 kb. The middle panel shows the same blot probed with a DRacB cDNA, which detects transcripts of 2.0 and 2.9 kb. The 2.0 kb transcript may be that of DRacA detected by cross hybridization. The bottom panel shows the blot probed with an actin cDNA as a control for loading.](image)

![Fig. 4. Expression of the N17DRacA transgene as a 1.8 kb transcript in adult flies of the four insertion lines following a 37°C heat shock. The uninduced levels of N17DRacA mRNA are shown by the siblings maintained at 22°C. The faint upper band (arrowhead) visible in the flies maintained at 22°C is probably the wild-type DRacA transcript. The bottom panel shows the blot reprobed with an actin cDNA as a control for loading.](image)
actin/myosin along the leading edge. Individuals only mildly affected by the induction of N17DRacA show an accumulation of these proteins along the leading edge (data not shown), whereas more strongly affected embryos show severe reductions relative to levels elsewhere in the epidermis (Fig. 8C-F). Induction of the WT-10 transgene had no effect on actin and myosin accumulations along the leading edge (data not shown). Panels C and D of Fig. 8 show an individual double labelled for actin and myosin, respectively. The patchy distributions of actin and myosin along the leading edge are very similar, in that they accumulate in the same areas. This colocalization of actin and myosin was found consistently in double labelled embryos. Panels E and F of Fig. 8 show individuals, stained for actin and myosin respectively, in which many epidermal cells have failed to undergo elongation. There is a tendency for actin and myosin to form clumps in these cells. The localization of actin to the membranes of the amnioserosa cells (seen in Fig. 8A), is disrupted by expression of N17DRacA (Fig. 8 C,E), an effect similar to that seen for α-spectrin (Fig. 7B). The actin and myosin staining patterns for N17-104, WT-10, and wild-type embryos maintained at 25°C were all indistinguishable from those shown in Fig. 8A and B (data not shown).

**DISCUSSION**

**Relationship of DRacA and DRacB proteins to Rac1 and Rac2**

We have identified two Drosophila genes, DRacA and DRacB, encoding proteins with strong homology to human Rac1 and Rac2. Although both Drosophila genes encode proteins of greater homology to Rac1 at the amino acid level, their cDNA sequences are more homologous to the Rac2 cDNA sequence. Thus, it is not clear that the two Drosophila genes represent the direct homologues of the two mammalian Rac genes, and they may be derived from a different duplication event. The very high homology between Drosophila and mammalian Rac proteins is consistent with the observation, derived from a phylogenetic analysis of the Rho subfamily, that the Rac proteins are evolving at a low rate (Vincent et al., 1992). The products of the Drosophila Rac genes may behave more like Rac1 than Rac2 as, like Rac1, they are probably expressed in a wide range of tissues.

**Drac1 and Drac2**

During the course of this work Luo et al. (1994) reported the isolation and characterization of a Drosophila Rac homologue encoded by a gene they termed Drac1. Drac1 is the same gene as DRacA. Luo et al. (1994) found that that Drac1 is widely expressed during development but greatly enriched in the mesoderm. We have not been able to detect a dramatic enrichment in the mesoderm in our RNA in situ hybridizations with DRacA. Luo et al. (1994) used the GAL4 system to investigate the potential role of Drac1 in muscle development, and found that when expressed in mesoderm cells, dominant inhibitory Drac1 mutant transgenes cause excessive myoblast fusion. The mutations used were N17, equivalent to N17DRacA, and L89, a serine to leucine change at amino acid 89 based on the dominant inhibitory F89 mutation in let-60, a gene encoding a Ras homologue in C. elegans (Beitel et al., 1990). Expression of an activated V12 mutant Drac1 transgene under the same conditions as the dominant inhibitory mutants causes a complete failure of myoblast fusion. The role of Drac1 in nervous system development was assessed by expressing the mutant transgenes in neurons using the GAL4 system. Both the L89 and V12 mutants cause axon outgrowth defects in the peripheral nervous system. Luo et al. (1994) have also isolated a second Drosophila Rac gene, Drac2, which is likely to be equivalent to DRacB, and a Drosophila homologue of CDC42 termed Dcdc42. When Dcdc42 transgenes were characterized in a similar set of studies as used on Drac1, it was found that...

![Fig. 5. Cuticle phenotypes of heat shocked embryos homozygous for the N17DRacA transgene.](image)
the Dcdc42 mutants cause different defects in neuronal and muscle development.

**Dominant inhibitory DRacA expression and dorsal closure**

The expression of N17DRacA during embryogenesis results in a high frequency of embryos with failures in dorsal closure that appear as scabs or holes in the dorsal cuticle. The cuticle in the vicinity of these defects is consistently buckled. This phenotype closely resembles that of mutations in coracle (cor), a gene encoding a Drosophila protein associated with septate junctions, which is homologous to the membrane-skeleton protein 4.1 (Fehon et al., 1994). Depending on the allele, embryos homozygous for cor mutations exhibit either a scab or hole on the dorsal side. The defects in dorsal closure seen after N17DRacA expression are accompanied by abnormalities in the cell shapes of the lateral epidermis. Cell shape defects have not been reported in cor mutants, but two other genes whose mutants show defects in the dorsal cuticle do show abnormalities in the shape of lateral epidermal cells. Embryos homozygous for mutations in the zip locus have an opening in the dorsal cuticle (Nüsslein-Volhard et al., 1984). The epidermal cells flanking the amnioserosa in zip mutants are abnormal in shape and disorganized, resembling what we see.

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**Fig. 6.** Embryos stained with anti-Fasciclin III antibodies. Anterior is to the left. (A) Dorsal view of a wild-type embryo during dorsal closure. (B) Detail of the epidermal cells flanking the amnioserosa of embryo in A. Fasciclin III is absent from the dorsal ends of these cells (arrowhead). (C) Dorsal view of a stage 16 wild-type embryo after dorsal closure. (D) Detail of the embryo in C, showing orderly arrangement of elongated cells. Fasciclin III is expressed at the dorsal midline where the cells have come together (arrowhead). (E) Dorsal view of stage 16 N17-104 embryo following 30 minute heat shock. There is a large opening in the dorsal side. (F) Detail of the lateral epidermis of the embryo in E. The cells are less organized and rounder than those in D. Note the patch of elongated cells (arrowheads). (G) Lateral epidermis of N17-104 embryo similar to that in E. The leading edge is irregular in shape and heavy Fasciclin III deposition is seen on some dorsal surfaces (arrowhead). (H) Dorsal view of N17-104 embryo heat shocked for 1 hour. There are two holes in the dorsal surface. Note that cells that have come together along the midline are not properly elongated (arrowheads). The dorsal surface of the head is open, exposing the brain (arrow).
in embryos following N17DracA induction (Young et al., 1993). Mutants in the gene puckered do not show appropriate cell shape changes around the amnioserosa and as a result the dorsal cuticle is buckled in a similar fashion to that of the heat shocked N17-104 embryos (Ring and Martinez Arias, 1993). puckered embryos do not have a dorsal hole or scab, however, and successfully complete dorsal closure. Thus, the cell elongations that occur in the lateral epidermis may not be essential for closure to proceed to completion (Ring and Martinez Arias, 1993), but are required for a normal, flexible dorsal surface that is able to withstand later movements in the embryo. There may be cases where dorsal closure occurs to completion in zip and N17-104 embryos but that the tightly fitting epidermis ruptures later.

Dominant inhibitory DRacA expression and the cytoskeleton
As mentioned above, the dorsal closure defects in zip mutants are similar to those generated by expression of N17DracA. The zip dorsal hole is believed to be caused by a lack of non-muscle myosin at the leading edge (Young et al., 1993). The phenotype is most like that of our mildly affected N17-104 embryos (see Fig. 7B), in that there is still a considerable degree of epidermal cell elongation. The extent of epidermal cell elongation in zip and N17-104 embryos is likely to be directly related to the amounts of myosin and actin along the leading edge, as it is myosin, acting as a molecular motor with a filamentous actin substrate, that is believed to be driving cell shape change in the lateral epidermis (Young et al., 1993). In zip mutant embryos only myosin is affected as they have a normal distribution of actin along the leading edge (Young et al., 1993). We have found that expression of N17DracA disrupts the accumulation of both myosin and actin along the leading edge. This may explain the extreme lack of epidermal cell shape change seen in some N17-104 embryos. Indeed, there is a good correlation between the quantities of myosin and actin at the leading edge and the degree of cell elongation in the lateral epidermis.

The normal accumulation of actin along the leading edge in zip mutants indicates that actin is not dependent on myosin for its localization to this region. However, myosin may require the presence of actin to localize to the leading edge as actin and myosin remain at shared areas on the leading edge in embryos in which N17DracA has been induced. In addition to actin and myosin, the localization of another cytoskeletal protein, α-spectrin, is disrupted following N17DracA induction. The expression of N17DracA causes the loss of α-spectrin from the leading edge and the membranes of the amnioserosa cells, a pattern paralleling the loss of actin. Pesacreta et al. (1989) have shown that during Drosophila embryogenesis α-spectrin occupies regions with previously existing accumulations of actin. All these results indicate that α-spectrin, like myosin, may require actin for its localization.

It is interesting to note that the induction of N17DracA does
not have a uniform effect on the distribution of actin along the membranes of the epidermal and amnioserosa cells. There are dramatic reductions in the accumulation of actin along the membranes of the amnioserosa cells and the leading edge only. With the exception of the dorsal sides of the leading edge cells, peripheral actin is not reduced in the epidermis, in fact there is an accumulation of actin along the epidermal cell membranes in individuals strongly affected by N17DRacA (Fig. 8E). This suggests that there may be a specific process involved in peripheral actin localization in the amnioserosa and the leading edge. What purpose, if any, the peripheral actin serves in the amnioserosa is unclear. It remains to be determined if the N17DRacA-induced loss of this actin, and the accompanying loss of α-spectrin, has an effect on the fate of the amnioserosa cells.

**Potential targets of dominant inhibitory DRacA**

The identity of the exchange factor(s) binding N17DRacA has

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**Fig. 8.** Confocal fluorescent micrographs of the boundary between the amnioserosa (top of each micrograph) and the epidermis during dorsal closure. Arrowheads indicate the leading edge. All embryos were heat shocked for 1 hour. Embryos were stained for filamentous actin with phalloidin or for nonmuscle myosin heavy chain with antibodies, or both. (A) Wild-type embryo stained for actin. There is an intense band of staining along the leading edge, and strong staining along the membranes of the amnioserosa cells. (B) Wild-type embryo stained for myosin. There is intense punctate staining along the leading edge. (C) N17-104 embryo stained for actin. Actin staining along the leading edge is patchy; the arrowheads indicate two areas of actin accumulation. The staining in the membranes of the amnioserosa cells is weak compared to A. (D) Same embryo as in C, double labelled for myosin. The myosin staining along the leading edge is similar to that of actin, with accumulations of myosin at spots shared with actin (arrowheads). (E) N17-104 embryo, stained for actin, in which no cell elongations are visible in the lateral epidermis. Actin staining in the amnioserosa cells and leading edge is severely reduced compared to wild-type, and there is clumpy actin staining along the membranes of the epidermal cells. (F) N17-104 embryo, stained for myosin, with poor elongation of epidermal cells. Myosin staining along the leading edge has been disrupted, and there are clumps of myosin staining along the membranes of the epidermal cells.
not been established. The CDC42 exchange factor dbl (Hart et al., 1991) has some activity for Rac as does smgDGS, which also stimulates exchange on Rho, Ki-ras and Rap1 (Hiraoka et al., 1992). N17Rac1, being incapable of blocking Rho-dependent induction of stress fibers by lysophosphatidic acid or bombesin, is not believed to be interacting with smgDGS (Ridley et al., 1992). The finding that Dcdc42 mutant proteins have effects on cell development distinct from analogous Drac1 mutant proteins, suggests that dominantly acting p21 mutants may be limited in the range of p21s they affect, and that the phenotypes they generate are a good reflection of the function of their wild-type counterparts (Luo et al., 1994). Additionally, comparison of the effects of activated and dominant inhibitory Rac indicates that dominant inhibitory Rac impairs wild-type Rac signalling (Ridley et al., 1992; Luo et al., 1994). Rac stimulates actin polymerization in a specific pattern in mammalian cells (Ridley et al., 1992). That Rac may also participate in actin localization during Drosophila development is supported by the finding that activated Drac1 mutant protein causes an abnormal accumulation of actin when expressed in neurons (Luo et al., 1994). Given the above considerations, it is highly likely that the Drosophila Rac proteins participate in the accumulation of actin at the leading edge during dorsal closure.

The Drosophila Rho subfamily and development

In addition to dorsal closure defects, N17DRacA induction can also produce abnormalities in germband retraction (Fig. 5F) and head involution (Figs 5E, 6H). Defects in head involution are also seen in cor embryos and zip embryos (Fehon et al., 1994, Young et al., 1993). It is likely that N17DRacA is disrupting requisite cell shape changes in these processes, although this has not been investigated. Studies on the distribution of myosin and actin during early embryonic development indicate other developmental events that the Rho subfamily may participate in. A myosin molecular motor, acting in conjunction with actin, is likely to function in cellularization and the cell shape changes of gastrulation (Young et al., 1991). Both myosin and actin accumulate at the leading edge of the furrow canals during cellularization, and cells that undergo a constriction during gastrulation have an accumulation of myosin and actin at the constricted end, similar to that seen along the leading edge during dorsal closure (Young et al., 1991). There is also evidence for the participation of myosin in the cell shape changes of imaginal disc morphogenesis (Young et al., 1993). Preliminary work indicates that expression of either a wild-type or a dominant inhibitory DRacA transgene during imaginal disc morphogenesis causes lethality (N. Harden, unpublished observations).

The results of the present study, along with the work of Luo et al. (1994) on the involvement of Rac and CDC42 in neuronal and muscle development, indicate that the Rho subfamily proteins have a diverse set of roles in Drosophila development. We have shown that during Drosophila embryogenesis, as in mammalian cell culture, expression of a dominant inhibitory Rac protein can affect cell morphology by disrupting the assembly of localized cytoskeletal structures. Our results implicate a Rho subfamily protein(s), most probably Rac, in an accumulation of cytoskeletal elements at one side of the epidermal cells of the leading edge. This is reminiscent of the polarization of yeast cells during bud formation, a process involving Cdc42Sc, the closest relative of Rac in yeast, which lacks Rac proteins (Adams et al., 1990, Johnson and Pringle, 1990). The parallels between our results and those studies done on single cells indicate that Drosophila will be an ideal system for investigating the role of the Rho subfamily proteins in the regulation of cell shape changes in developing tissues. In addition to such structural investigations, the phenotypes caused by defects in cell morphology should be useful in a genetic analysis of signal transduction by the Rho subfamily.

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