DRacGAP, a novel Drosophila gene, inhibits EGFR/Ras signalling in the developing imaginal wing disc

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Accepted 5 October; published on WWW 14 November 2000

SUMMARY

We have identified a novel Drosophila gene, DRacGAP, which behaves as a negative regulator of Rho-family GTPases DRac1 and DCdc42. Reduced function of DRacGAP or increased expression of DRac1 in the wing imaginal disc cause similar effects on vein and sensory organ development and cell proliferation. These effects result from enhanced activity of the EGFR/Ras signalling pathway. We find that in the wing disc, DRac1 enhances EGFR/Ras-dependent activation of MAP Kinase in the prospective veins. Interestingly, DRacGAP expression is negatively regulated by the EGFR/Ras pathway in these regions. During vein formation, local DRacGAP repression would ensure maximal activity of Rac and, in turn, of Ras pathways in vein territories. Additionally, maximal expression of DRacGAP at the vein/intervein boundaries would help to refine the width of the veins. Hence, control of DRacGAP expression by the EGFR/Ras pathway is a previously undescribed feedback mechanism modulating the intensity and/or duration of its signalling during Drosophila development.

Key words: RacGAP, Rac GTPase, Signal transduction, Apoptosis, EGFR, Drosophila melanogaster, Imaginal disc

INTRODUCTION

The development of multicellular organisms depends, to a large extent, on external stimuli controlling a variety of cellular processes, such as cell growth and proliferation, cell survival, and cell determination and differentiation. These stimuli, in the form of extracellular ligands, interact with and activate or repress transmembrane receptor proteins. The Ras superfamily of small GTPases, which includes among others the Ras and Rho families, links receptor stimulation to activation of cytosolic protein kinase cascades which, in turn, orchestrate cytoplasmic and nuclear events (Bourne et al., 1990).

The mammalian Rho-family comprises at least 14 different proteins; the best characterised are RhoA, Rac1 and Cdc42Hs (reviewed by Van Aelst and D’Souza-Schorey, 1997). These proteins act as signal transducers by switching between inactive, GDP-bound and active, GTP-bound forms. Their activity is regulated by the opposing effects of guanine nucleotide exchange factors (GEF), positive regulators that enhance the exchange of bound GDP for GTP, and GTPase-activating proteins (GAP), negative regulators that stimulate the intrinsically low GTPase activity of the Rho proteins. They are further regulated by guanine-nucleotide dissociation inhibitor proteins which stabilise the bound GTP. RhoGEFs and RhoGAPs, by analogy to RasGEFs and RasGAPs, are thought to be the immediate targets of activated receptors (Boguski and McCormick, 1993).

Mammalian Rho-family GTPases regulate cell proliferation and also many actin-based cellular processes such as cell motility, axonal growth and guidance, formation of cell adhesion complexes, intracellular membrane trafficking and cytokinesis (reviewed by Hall, 1998). They participate in transcriptional regulation, mainly through activation of the c-Jun NH2-terminal kinase (JNK) and p38 mitogen-activated (MAP) kinase cascades (Van Aelst and D’Souza-Schorey, 1997).

Homologues to the Rho-family GTPases and their regulators have been identified in D. melanogaster (DRhoA, DRhoL, DRac1, and DCdc42, DRhoGAP1 and 2, pebble and RotundRacGAP (RnRacGAP), reviewed by Van Aelst and D’Souza-Schorey, 1997). This has facilitated the genetic analysis of their physiological roles. In accordance with their function in reorganising the actin cytoskeleton, Drosophila Rho-family proteins control growth cone guidance (reviewed by Luo et al., 1997), myogenesis (Luo et al., 1994), remodelling of synaptic terminals (Sone et al., 1997) and cytokinesis (Prokopenko et al., 1999). They also participate in gastrulation (Barrett et al., 1997; Häcker and Perrimon, 1998), embryonic segmentation (Magie et al., 1999) and cell migration (reviewed by Montell, 1999). Epithelial planar polarity (EPP) and embryonic dorsal closure depend both on actin cytoskeleton reorganisation and JNK activation, which are controlled by Rho-family proteins (reviewed by Eaton, 1997; Noselli and Agnès, 1999). Only one putative RacGAP, RnRacGAP, has been identified in Drosophila (Agnel et al., 1992). Its ectopic expression in cellularising embryos disrupts the actin cytoskeleton and its overexpression in pupae slightly alters the pattern of wing veins, wing shape and EPP (Guichard et al., 1997).
Initially, each of the components of the Ras superfamily were thought to control different, independent regulatory pathways. However, nowadays there is compelling evidence of crosstalks among their different signalling pathways (Schwartz and Baron, 1999). For example, in mammalian cells in culture, Ras and Rho-family proteins cooperate in the control of cell proliferation. Thus, activated forms of Rho or Rac synergise with Raf, a downstream kinase of Ras, in focus-formation assays, and dominant negative forms of Rho, Rac and Cdc42 inhibit cell-transformation caused by oncogenic Ras (reviewed by Van Aelst and D’Souza-Schorey, 1997). Here we present, to our knowledge for the first time, evidence for the cooperation of Rac and Ras signalling pathways in the context of a whole organism. We describe a novel Drosophila gene, DRacGAP, which encodes a putative GAP for Rac and Cdc42 GTPases. We show that both DRacGAP and Drac1 are involved in the control of cell proliferation. Moreover, reduced activity of DRacGAP or overexpression of DRac1 in the wing imaginal disc cause similar defects: widening of veins, development of extra sensory organs (SOs), apoptosis and appearance of enlarged cells that differentiate multiple hairs. Indeed, this pathway controls the expression of its own negative and positive regulators (Wasserman and Freeman, 1994) controls multiple developmental processes (Rommel and Hafen, 1998; Hackel et al., 1999) and is accurately regulated. Interestingly, expression of DRacGAP is repressed by EGFR/Ras signalling in the prospective veins and accumulates below) in the pUAST vector (Brand and Perrimon, 1993). The subcloning wild-type Drosophila pathway during provides a new mechanism to modulate the intensity of this cascade (Brunner et al., 1994; Díaz-Benjumea and Hafen, 1999). Interestingly, expression of DRacGAP cDNA was subcloned in A 0.2 kb cDNA containing the entire DRacGAP ORF was fortuitously isolated from a Drosophila cDNA imaginal disc library (provided by J. Botas) as a cloning artefact joined to a kachnan cDNA clone (Sotillos et al., 1997) and subcloned into pBluescript as an EcoRI fragment. The isolated DRacGAP cDNA was full length as its size coincides with that of DRacGAP mRNA detected by northern blot (not shown). cDNA was sequenced with the ABI Taq cycle sequencing system and analysed with the University of Wisconsin GCG software package (Devereux et al., 1984). GenBank accession number of DRacGAP is AI251502.

Comparison of DRacGAP cDNA and corresponding genomic sequences, contained in bacteriophage PID504160 (mapped at 50C6-8, Berkeley Drosophila Genome Project), revealed that the DRacGAP gene contains 4 exons separated by three introns of 69, 63 and 513 nucleotides.

MATERIALS AND METHODS

Drosophila stocks

The strains used in this study are: Df(2R)CX1, which uncovers the DRacGAP gene; Df(3R)I65, which uncovers the pRacGAP gene; Rho GTase pathway alleles and transgenes: RhoA and UAS-Rho (Strutt et al., 1997); UAS-DRac1, UAS-DRac1N17, UAS-Drac427 and UAS-Drac427V12 (Luo et al., 1994), Cdc42 (Fehon et al., 1997), DRho-GEF2 (PZ 04291, Hämker and Perrimon, 1998), Pak4 (Hing et al., 1999) and Pkn5 (PZ 06736, Lu and Settleman, 1999); EGFR pathway alleles and transgenes (veinlet7 (ve7), vein1 (vn), UAS-RasV12 (Karin and Rubin, 1998), UAS-Raf9N3 (Martín-Blanco et al., 1999); UAS-rolledSem (Martín-Blanco, 1998), UAS-argos (UAS-aro and aor8); a gift from M. Freeman) and UAS-p35 (Hay et al., 1994), UAS-p21 (Karin and Rubin, 1998), UAS-Cyclin (Weigmann et al., 1997), UAS-string (Neufeld et al., 1998); Act5CFTDRaf FRTnuc-lacZ (Struhl and Basler, 1993; y Act5CRTCdc2FRTGa4, (a gift from E. Sánchez-Herrero), neutralized-lacZ (A101-IF3), en-4,4), Scallop-lac4 (Sd4-Ga4), 69B-Ga4, MS1096-Ga4, dpp-lac8-Ga4 and C765-Ga4. They are described in FlyBase (http://flybase.bio.berkeley.edu/7081). Transgenic lines UAS-DRacGAP, UAS-DRacGAP(y118) and UAS-DRacGAP(D) were generated by subcloning wild-type DRacGAP cDNA or the mutated forms (see below) in the pUAST vector (Brand and Perrimon, 1993). The resulting pUAST-plasmids were used to transform wfl118 embryos (Ashburner, 1989).

Overexpression experiments

Different UAS transgenic lines were crossed with several Gal4 lines and larvae were raised at 18°C, 25°C or 29°C. An en-G4/S66a TM6b/UAS-DRacGAP(D) stock was maintained at 18°C and used to test modifications of the DRacGAP(D) phenotype in different genetic backgrounds.

Overexpression of UAS transgenes was also carried out by the flip-out method (Struhl and Basler, 1993) in larvae of the genotype y w hs-FLP; ActFRtcdc2FRTGa4; UAS-y+/UAS-DRacGAP(D). Expression of the Flipase (FLP) gene was induced at first, second and third instar larvae by 1 hour incubation at 37°C. Clones were distinguishable as y*.

Molecular characterisation of DRacGAP

A 2.3 kb cDNA containing the entire DRacGAP ORF was fortuitously isolated from a Drosophila cDNA imaginal disc library (provided by J. Botas) as a cloning artefact joined to a kachnan cDNA clone (Sotillos et al., 1997) and subcloned into pBluescript as an EcoRI fragment. The isolated DRacGAP cDNA was full length as its size coincides with that of DRacGAP mRNA detected by northern blot (not shown). cDNA was sequenced with the ABI Taq cycle sequencing system and analysed with the University of Wisconsin GCG software package (Devereux et al., 1984). GenBank accession number of DRacGAP is AI251502.

Site directed mutagenesis

A 0.2 kb SacII-EcoRV from DRacGAP cDNA was subcloned in pBluescript and used as template for inverted PCR-mutagenesis essentially as described by Hemsley et al. (1989). Oligonucleotides used to generate the EIE deletion were GCCCGCGGCCTGACCGA and ATTTACGGTGCAACACTGTG and those used for site directed mutagenesis of arg147 were CAGTGTTCCTCGTCCGA-GCC and ATAGAGGCAACCTCGGTCAG. The arg codon was replaced by the sequence shown in bold. The mutated fragments were excised from pBluescript and inserted in place of the wild-type fragments in subclones of the DRacGAP cDNA in pGEM1. Mutations were confirmed by sequencing.

In situ hybridisation and immunocytochemistry

In situ hybridisation with digoxigenin-labelled probes, combined with X-gal staining, anti-β galactosidase (Promega) and phalloidin (Molecular Probes) stainings are described by Cubas et al. (1991), TUNEL labelling by Milán et al. (1997), staining with anti-double phosphorylated-ERK (dp-ERK, Sigma), to determine the pattern of activated MAPK, by Martín-Blanco et al. (1999) and in situ hybridisation to polythene chromosomes by Ashburner (1989).

Generation of β-galactosidase expressing clones

y w hs-FLP; en-G4/S66a TM6b/UAS-DRacGAP(D) females were crossed with Act5CFTDRaf FRTnuc-lacZ males (Struhl and Basler, 1993). Larvae were heat-shocked for 7 minutes at 37°C at 24-48 hours after egg laying to induce expression of the FLP gene. After 3 days at 25°C, lacZ-expressing cells in the wing discs were identified by anti-β-galactosidase staining.

Other methods

Basic molecular biology techniques were carried out as described by Sambrook et al. (1989). Statistical analyses were those described by Zar (1999).
RESULTS

Molecular characterisation of the DRacGAP gene

We have isolated a D. melanogaster cDNA clone that encodes a protein of 625 amino acids whose sequence is closely related to RacGAPs from different organisms (Fig. 1A). This protein contains a domain (residues 395-537) including the three conserved amino acid blocks and the arginine finger that define the GAP domain in GAPs for Rho-family GTPases (Ahmed et al., 1994; Scheffzek et al., 1998). It also presents two motifs usually found in these proteins (Lamarche and Hall, 1994) – a proline rich region, putative recognition motif for SH3-domain containing proteins, and a cysteine-rich domain, similar to the diacylglycerol-binding domain of protein kinase C. The putative GAP domain is most similar to those of Drosophila RNacGAP (Agnel et al., 1992), a mouse RacGAP (Wooltorton et al., 1999) and human n-chimaerin (Ahmed et al., 1994), which is a GAP specific for Rac GTPase (Dieckmann et al., 1991). Accordingly, and also based in the genetic interactions described below, we have named this gene DRacGAP. The DRacGAP gene was mapped to cytological region 50C4-8 by in situ hybridisation (not shown).

Expression pattern of DRacGAP

DRacGAP gives rise to a unique transcript of 2.3 kb present throughout development (not shown). The expression pattern of DRacGAP is highly dynamic. It is ubiquitous during the initial stages of embryogenesis and becomes restricted, after germ band retraction, to the central and peripheral nervous system (not shown). In the early second instar wing disc, DRacGAP mRNA accumulation is widespread expression occurs mostly in the presumptive wing region (Fig. 1C and not shown). In the early second instar wing imaginal disc, DRacGAP is expressed in the eye-antenna disc in two stripes of cells located at, and ahead of, the morphogenetic furrow and in SH3-domain containing proteins, and a cysteine-rich domain, similar to the diacylglycerol-binding domain of protein kinase C. The putative GAP domain is most similar to those of Drosophila RNacGAP (Agnel et al., 1992), a mouse RacGAP (Wooltorton et al., 1999) and human n-chimaerin (Ahmed et al., 1994), which is a GAP specific for Rac GTPase (Dieckmann et al., 1991). Accordingly, and also based in the genetic interactions described below, we have named this gene DRacGAP. The DRacGAP gene was mapped to cytological region 50C4-8 by in situ hybridisation (not shown).

Analysis of DRacGAP function in the wing imaginal disc

The pattern of expression of DRacGAP in third instar wing discs prompted us to examine its role in vein patterning. Since there are no DRacGAP mutants available, we used the Gal4/UAS system (Brand and Perrimon, 1993). First, we established transgenic flies carrying wild-type DRacGAP under the control of the UAS element (UAS-DRacGAP lines). Overexpression of DRacGAP in the wing disc interfered with vein development. Thus, overexpression driven by the Gal4 lines MS1096 or C765 eliminated the corrugation of the adult veins (Fig. 2A,B and not shown). Overexpression driven by en-G4 reduced the expression of a vein marker gene in the wing disc (Fig. 2D) and resulted in thinning of adult veins (not shown). A reduction in wing size and a decrease in the number of SOs were also found (Fig. 2B and Table 1).

To investigate whether a reduction in DRacGAP function would cause reciprocal effects, we generated two types of transgenic flies expressing dominant negative (DN) forms of DRacGAP under the control of UAS. In one of them, the EIE motif (amino acids 405-407) was deleted (UAS-DRacGAPΔEIE line). Removal of the corresponding amino acids in human n-chimaerin abolishes its RacGAP activity without impairing its ability to bind to Rac (Ahmed et al., 1994). In the other DN form, the conserved arginine residue of the arginine finger (arg417) was replaced by glutamine (UAS-DRacGAPR417Q line). The homologous arginine residues of RasGAP and Rhogap arginine fingers are essential for their ability to increase the hydrolysis of GTP bound to the corresponding GTPase (Scheffzek et al., 1998). Hence, both DN proteins should compete with the wild-type protein for binding to the GTPase and, when bound to it, should maintain it in the GTP-bound active state, effectively mimicking a reduction in DRacGAP function.

Wings of flies expressing UAS-DRacGAPΔEIE in the wing disc driven by en-G4 (referred to hereafter in the text as DRacGAPDN flies) displayed loss of wing tissue (wing notching), veins of increased width, fusion of adjacent veins and appearance of vein material connecting neighbouring veins (Fig. 2H). In addition, they had extra SOs along the vein L3 and in the proximal regions of veins L4 and L5 (Fig. 2J, and Table 1). DRacGAPDN wings showed enlarged cells (indicated by low trichome density, Fig. 2H) and alteration in the number and polarity of the trichomes (Fig. 2I). The enlarged cells were also visible in the imaginal disc (phalloidin staining, which labels cortical actin; Fig. 2K,L) indicating the early onset of

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<th>Genotype</th>
<th>Wing phenotype (%)</th>
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<td></td>
<td>Weak</td>
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<tr>
<td>MS1096; UAS-DRacGAP/+</td>
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<tr>
<td>en-G4/+; UAS-DRacGAPΔEIE/+</td>
<td>20</td>
</tr>
<tr>
<td>en-G4/+; UAS-DRacGAPR417Q/+</td>
<td>85</td>
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<tr>
<td>en-G4/+; UAS-DRacGAPR417Q/+</td>
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<td>en-G4/+ UAS-DRacGAPΔEIE/+</td>
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Wing phenotypes were classified as weak (presence of enlarged cells with polarity defects, as shown in Fig. 2G), intermediate (showing in addition widened veins) and strong (with additional wing notching, as shown in Fig. 2H). Number of sensilla are the average number of campaniform sensilla in the L3, L4 and L5 veins and anterior crossvein (x.e.m.). Wild-type wings only show four campaniform sensilla on the L3 vein and one on the anterior crossvein. n, number of wings examined; T, temperature (*C); n.d., not determined. (*,‡,§) Differences between the indicated values are statistically significant (P<0.05 in all comparisons; Mann-Whitney U-Test).
this defect. Overexpression of UAS-DRacGAP<sup>R417Q</sup> produced the same effects, although they were weaker than with UAS-DRacGAP<sup>DIE</sup> (Fig. 2G and Table 1). Accordingly, all genetic interactions shown below were performed using the UAS-DRacGAP<sup>DIE</sup> transgene. Overexpression of either UAS-DRacGAP<sup>DN</sup> transgene with other Gal4 lines (MS1096, 69B) or in clones of Gal4-expressing cells (see Materials and methods) caused the same phenotypes (not shown).

All the defects of DRacGAP<sup>DN</sup> flies were rescued by coexpression of wild-type DRacGAP (Fig. 3A,B), while remaining unaltered in the presence of UAS-lacZ, indicating that they result from reduced DRacGAP activity. Moreover, the mutant phenotype of DRacGAP<sup>DN</sup> flies was increased in flies with only one copy of the endogenous DRacGAP gene (compare Figs 3C and 2H; 100% of the flies presented the phenotype showed in Fig. 3C). Still, DRacGAP<sup>DN</sup> proteins may interfere to some extent with the function of the related RnRacGAP, since heterozygosity for RnRacGAP (in Df(3R)dsx 10M/+ flies) moderately increased the loss of wing tissue of DRacGAP<sup>DN</sup> flies (compare Figs 3D and 2H).

DRac1 and Dcdc42 are putative targets of DRacGAP

The above results suggest that the small GTPase(s) whose activity is downregulated by DRacGAP should be required for vein and SO development and for cell proliferation. By analogy with its closest homologues, DRacGAP should regulate RacGTPase. Indeed, we found that overexpression of wild-type DRac1 (UAS-DRac1) driven by en-G4 caused vein thickening.
DRacGAP inhibits EGFR/Ras pathway

Fig. 2. Functional analysis of DRacGAP. (A-D) Overexpression of DRacGAP interferes with vein and SO development and reduces wing size. (A) Dorsal view of a wild-type wing showing longitudinal veins L3 and L4 and one of the campaniform sensilla of L3 (arrow). (B) A similar region of a MS1096; UAS-DRacGAP wing shows loss of the dorsal corrugation of L3 and L4 veins and of campaniform sensilla (arrow). Note the reduction in the distance between veins L3 and L4. (C-E) ve/rho expression responds to the Rac pathway. Late third instar wing discs from wild-type (C), en-G4/+; UAS-DRacGAP/+ (D) and en-G4/+; UAS-DRacGAPDEIE/+ (E) larvae showing that expression of ve/rho in veins and wing margin (arrowheads and arrow, respectively) is reduced (D) or expanded (E) in the posterior compartment. (G-L) Wing phenotypes of flies with reduced DRacGAP activity. (F) Wild-type wing. (G-J) Flies overexpressing UAS-DRacGAPR417Q (G) or UAS-DRacGAPDEIE (H-J) driven by en-G4 show veins of increased width and fusion of veins (arrowhead in H), loss of wing tissue (H) and enlarged cells (arrows in G and H). (L) Magnified views of en-G4/+; UAS-DRacGAPDEIE/+ wings. Note in I the development of enlarged cells, each one possessing several abnormally oriented trichomes (arrowhead, normal trichome orientation is indicated by the arrow) and in J the extra SOs (campaniform sensilla, some are indicated by arrows) along the L3 vein. (See also Table 1). (K,L) Anterior (K) and posterior (L) compartments of the same en-G4/+; UAS-DRacGAPDEIE/+ wing disc stained with FITC-phalloidin. Note cells of increased size in the posterior compartment (arrows). Flies were raised at 25°C (A, C-I, K,L) or 29°C (B, J). A, anterior compartment; P, posterior compartment. Line in F marks the anteroposterior compartment boundary.
Fig. 3. DRacGAPDN protein interferes with DRacGap function. Wing phenotypes of en-G4; UAS-DRacGAPΔEIE flies (A) are reversed by coexpression of UAS-DRacGAP (B) and severely or moderately enhanced in flies heterozygous for Df(2R)CX1 (C) and Df(3R)dsx10M (D) (compare C and D with the en-G4/+; UAS-DRacGAPΔEIE/+ wing raised at 25°C shown in Fig. 2H). Heterozygous Df(2R)CX1/+ or Df(3R)dsx10M/+ flies are phenotypically wild type (not shown). Flies were grown at 29°C (A,B) or 25°C (C,D). Arrowheads in C,D indicate enlarged veins. Wings are shown with anterior up and distal to the right. All wings are shown at the same magnification.

D RacGap and DRac1 affect the EGFR/Ras signalling pathway

As shown above, overexpression of UAS-DRacGAPDN or UAS-DRac1 caused vein enlargement and the appearance of extra Sos, two structures requiring EGFR/Ras/Raf/MAPK activity (Clifford and Schüpbach, 1989; Díaz-Benjumea and García-Bellido, 1990; Díaz-Benjumea and Hafen, 1994). Since Rac and Ras pathways cooperate in mammalian cells in the control of cell proliferation (see Introduction), we investigated whether the phenotypes associated to increased Rac signalling could be due to overactivity of the Ras pathway. This appears to be the case, since a reduction in EGFR signalling (by expression of UAS-RafDN, Martín-Blanco et al., 1999) reduced their vein, wing notching and large cell phenotypes (compare Fig. 5B,D with Fig. 4G,A, respectively). Similarly, when levels of the EGFR activators ve/rho (Sturtevant et al., 1993) and vein (vn; Schnepf et al., 1996; Simcox et al., 1996) were decreased (in ve7M vn/+ flies), the wing notching associated with DRacGAPDN expression was substantially corrected (Table 1). In contrast, activation of EGFR signalling enhanced the mutant phenotype of DRacGAPDN flies. Thus, although flies heterozygous for argos (aos+/+), a repressor ligand of EGFR (Freeman et al., 1992; Schweitzer et al., 1995), are phenotypically wild type, its combination with DRacGAPDN significantly increased the number of campaniform sensilla of DRacGAPDN flies (Table 1).

Note that the loss of vein tissue and reduced size of en-G4; UAS-RafDN wings were partially rescued by the expression of either UAS-DRacGAPDN or UAS-DRac1 (Fig. 5A-D). These results further suggest that the efficacy of the EGFR pathway was enhanced by increased Rac activity. Signalling by EGFR/Ras leads to phosphorylation and consequent activation of MAPK (Gabay et al., 1997). Rac signalling also ultimately activated MAPK since expression of UAS-DRacGAPDN widened the domains of accumulation of dp-ERK in the presumptive veins and spread them into the interveins (Fig. 5E,F). This effect was enhanced by coexpression of UAS-DRac1 (Fig. 5G). The activation of the EGFR pathway was also visualised by the increased expression of the downstream gene ve/rho (Martín-Blanco et al., 1999) (Fig. 2E). As expected, overexpression of DRacGAP produced the opposite effect (Fig. 2D).

Expression of DRacGAP appears to be decreased in the domains of EGFR activation. This is most apparent in the notum region of second instar wing disc (Fig. 1B), where the EGFR activator vein is expressed (Simcox, 1997), and in the presumptive veins of third instar wing disc (Fig. 1E), territories of maximal EGFR signalling (Gabay et al., 1997). This observation suggests that the EGFR pathway may repress the expression of DRacGAP. In agreement with this notion, expression of DRacGAP was either decreased or enhanced in cells expressing UAS-RasV12 or UAS-aos (Fig. 5I-K) in which the EGFR/Ras pathway is activated (Karim and Rubin, 1998) or repressed (Freeman et al., 1992; Schweitzer et al., 1995), respectively.

Overexpression of DRacGAPDN induces apoptosis

The above results suggest a cooperation between Rac and Ras signalling pathways in the activation of the MAPK. In the context of this cooperation, we analysed the other phenotypes of DRacGAPDN flies.

Apoptotic cells are rare in wild-type wing discs (Milán et
However, they were very abundant in DRacGAPDN discs (Fig. 6C), indicating that the loss of tissue in DRacGAPDN wings (Figs 2H, 6A) was due to apoptosis. Moreover, this loss was rescued by coexpression of the baculovirus caspase inhibitor p35 (Hay et al., 1994; Fig. 6D,F). The remaining DRacGAPDN phenotypes were not rescued, indicating that they are not indirect consequences of apoptosis.

Viability of the DRacGAPDN cells was monitored by clonal analysis. Lineage clones (labelled with lacZ) were induced in DRacGAPDN first instar larvae and were visualized 72 hours later. Clones were significantly more frequent in the A compartment of the presumptive wing (51 A clones and 14 P clones were found in 28 discs). This difference in clone frequency indicates a lower viability of DRacGAPDN-expressing cells, which should lead to their gradual loss from the disc epithelium by ‘cell competition’ (Simpson, 1979) and/or the apoptosis of the DRacGAPDN cells.

**DRacGAPDN caused cell cycle arrest**

DRacGAPDN wings displayed enlarged cells (Figs 2I, 6B) and showed abnormal EPP manifested by the appearance of several randomly orientated trichomes per cell (Fig. 2I). Enlarged cells may result from cell cycle arrest, since blocking cell cycle progression does not impair cell growth (reviewed by Conlon and Raff, 1999). To analyse whether DRacGAPDN cells were arrested at the G1 or G2 phases of the cell cycle, we attempted to normalise their size by expressing either Cin(3)E or string, which drive progression from G1 to S and G2 to M, respectively (Edgar and O’Farrell, 1990; Knoblich et al., 1994). Reversion to normal cell size was found only by expressing UAS-CycE (Fig. 6H), suggesting arrest at G1. Cell death and the development of multiple hairs per cell were similarly rescued, whereas the vein phenotype was not modified (Fig. 6H).

As indicated above, the efficacy of the Ras pathway seems to be enhanced in DRacGAPDN flies. Overactivation of the Ras pathway may also contribute to the G1 arrest of DRacGAPDN cells since we have found that although at 18°C overexpression of UAS-rolledsem (the activated form of MAPK) or UAS-DRacGAPDN did not enlarge cells or alter EPP, such modifications were observed upon coexpression of both genes (not shown). Although Ras is an inducer of cell proliferation, in mammalian cells, prolonged, high-intensity Ras/Raf signalling arrests cells in G1 (Sewing et al., 1997; Woods et al., 1997). This is mediated by Raf-dependent activation of the cyclin-dependent kinase (cdk) inhibitor p21WAF1/CIP1, which, indirectly, represses CycE transcription (Downward, 1997). Indeed, expression of UAS-p21WAF1/CIP1 in the imaginal wing disc caused mutant phenotypes somewhat similar to those of DRacGAPDN, namely, the appearance of enlarged cells with altered hair polarity (Karim and Rubin, 1998 and Fig. 6I) and apoptosis (not shown). As expected, these phenotypes were partially reversed by UAS-CycE overexpression (not shown), confirming that p21 arrests cells in G1 (de Nooij and Hariharan, 1995). Remarkably, expression of wild-type UAS-DRacGAP significantly rescued the mutant phenotype caused by expression of UAS-p21WAF1/CIP1 (Fig. 6J), suggesting a reduction in p21 activity associated with

Fig. 4. DRacGAP reduces DRac1 pathway activity. (A,B) Reduced activity of DRacGAP increases DRac1 signalling. (A) Overexpression of UAS-DRac1 driven by en-G4 at 18°C (A) causes wing phenotypes similar to those of en-G4/+; UAS-DRacGAPE116/+ flies (G). (B) Mutant phenotype is enhanced by coexpression of both transgenes (see also Table 1). Note the extensive vein thickening (arrowheads). (C-F) In contrast, overexpression of DRacGAP reduces DRac1 activity. Lethality of Sd-G4/+; UAS-DRac1/+ flies is rescued by coexpression of UAS-DRacGAP (D) and the reduced size of Sd-G4/+; UAS-DRac1M17/+ wings (E) is enhanced by expression of UAS-DRacGAP (F). Sd-G4/+; UAS-DRac1/+ flies raised at 18°C are phenotypically wild type. DRacGAPDN phenotypes (G) are partially rescued when one dose of Pak is eliminated (H, see also Table 1). Flies were grown at 18°C (A-F) and 25°C (G-H). All wings are shown at the same magnification.
decreased Rac activity. Since transcription of UAS-p21 is driven by the heterologous UAS promoter, this suggests that DRacGAP may be involved in posttranslational modification of p21. Interestingly, involvement of Rho in p21 stability has been described in cultured mammalian cells (Olson et al., 1998).

**DISCUSSION**

The activity of the signalling pathways that operate during development must be tightly regulated to achieve different thresholds required for the different cellular responses. An initial weak activation of a pathway could lead to transcription of activators of the pathway and/or repression of its inhibitors, to gain further amplification of the signal. During *Drosophila* vein development both positive (ve/rho) and negative (argos, sprouty and kekkon) regulators of the EGFR/Ras pathway are transcribed in response to the pathway (Perrimon and McMahon, 1999). In this report we show that signalling downstream of Rho-family GTPases cooperate with the EGFR/Ras pathway in activation of the MAPK. Furthermore, expression of DRacGAP, a negative regulator of Rho-family GTPases, is suppressed by EGFR/Ras signalling in the prospective vein regions of the wing disc. This transcriptional repression contributes to upregulation of the EGFR/Ras pathway in these regions thus providing an additional control level of the pathway.

**Function of DRacGAP in the wing disc**

The functional analysis of DRacGAP, performed by overexpressing wild-type and dominant negative forms, indicates that it negatively regulates cell proliferation and vein and SO development. It cannot be ruled out completely that DN forms of DRacGAP may also compete for targets with other RacGAPs. In fact, reducing the dosage of the related RnRacGAP (and of other uncharacterised genes included in the chromosomal deficiency used) slightly enhanced the phenotype of DRacGAP^DN flies. In any case, the functions of those other uncharacterised RacGAPs appear to be redundant.
with that of DRacGAP, since their putative reduction of activity is compensated for by increasing the amount of wild-type DRacGAP.

The GAP domain of DRacGAP is highly similar to that of n-chimaerin (Ahmed et al., 1994), a mammalian GAP specific for Rac GTPase (Dieckmann et al., 1991). This suggests that DRacGAP acts specifically on DRac GTPases, of which three are already known in *Drosophila* (Van Aelst and D’Souza-Schorey, 1997). In agreement with this notion, DRacGAP reduces DRac signalling while coexpression of wild-type *UAS-DRac1* and *UAS-DRacGAP* had a synergistic effect. Our results suggest that DRacGAP may also modify the activity of the Cdc42 GTPase. However, the possibility remains that a GAP specific for Cdc42 downregulates it. In that case, the observed genetic interactions between *DCdc42* and *DRacGAP* would indicate that both DRac and DCdc42 cooperate in the enhancement of the EGFR/Ras pathway, probably through the activation of a common downstream kinase effector (Fig. 7 and see below). Indeed, it is quite common for both GTPases act coordinately in the same developmental processes (reviewed by Van Aelst and D’Souza-Schorey, 1997).

**Cooperation of Rac and Ras pathways in cell proliferation**

The enlarged size of *en-G4; UAS-DRac* wings and the small size of wings with reduced Rac function indicate a role of DRac in cell proliferation control. In mammalian cells, Ras and Rac pathways cooperate in stimulating cell proliferation (Van Aelst and D’Souza-Schorey, 1997). We have found that, similarly, *Drosophila* Rac1 and Ras also appear to cooperate in this process since the reduced size of the wings of *UAS-Raf*-*DN* expressing flies is largely normalised by reduction of DRacGAP function or by overactivity of DRac1. The induction of cell death by *DRacGAP*-*DN*, where DRac activity is upregulated, is in apparent contradiction with these results. However, note that in mammalian cells, quantitative variations in the level of Ras signalling can cause very different effects (Marshall, 1995). Thus, instead of cell proliferation, high levels of Ras signalling may induce cell cycle arrest at G1 and apoptosis as part of a cell self protective mechanism (Downward, 1998). In that situation, G1 arrest is caused by Raf-dependent induction of p21 expression (Sewing et al., 1997; Woods et al., 1997) which inhibits Cdk activity and indirectly represses CycE transcription (Downward, 1997). The situation appears to be very similar in *Drosophila*. We have found that cell death of *DRacGAP*-*DN* flies could be attributed to their arrest at the G1 stage, since it is rescued by expression of *CycE*. Moreover, the accumulation of p21 causes apoptosis, enlargement of cell size and polarity defects (see also Karim and Rubin, 1998), phenotypes which are corrected by expression of *CycE*. Accordingly, we...
hypothesise that induction of cell cycle arrest and apoptosis by overactivity of the Rac pathway could be a consequence of increased Ras signalling. Rac would potentiate the reduced Raf signalling occurring in Raf\textsuperscript{DN} flies, allowing wing disc cells to proliferate, but in the presence of wild-type Raf, overactivity of Rac would enhance Raf signal to such a high level as to induce p21 expression and ultimately, apoptosis, as previously shown to occur after expression of constitutively active Ras (Karim and Rubin, 1998). This interpretation is supported by the partial rescue of the wing notchting phenotype of DRacGAP\textsuperscript{DN} flies in a ve vh heterozygous background and in Raf\textsuperscript{DN} flies where EGFR/Ras signalling is reduced.

Note that cell size and polarity phenotypes of DRacGAP\textsuperscript{DN} are similarly induced by p21 and reversed by CycE expression, indicating they can be attributed to cell cycle arrest. Indeed, it is known that cell cycle arrest may cause cell enlargement (Neufeld et al., 1998; Weigmann et al., 1997; reviewed by Conlon and Raff, 1999). Moreover, Drosophila mutations affecting cell cycle machinery are very frequently associated with defects in the development and polarity of the wing hairs (Weigmann et al., 1997).

Cross regulatory interactions between Rac and Ras pathways

The effects on vein and SO development caused by increased DRac1 activity are very similar to those associated with overactivity of the EGFR/Ras pathway (Baker and Rubin, 1992; Brunner et al., 1994; Karim and Rubin, 1998; Sturtevant et al., 1993; J. Culí, E. Martín-Blanco and J. Modolell, personal communication). We show that both pathways cooperate to implement these cell fates. Thus, the appearance of thickened veins and extra SOs in DRacGAP\textsuperscript{DN} flies was enhanced or suppressed by increased or decreased activity of the EGFR/Ras pathway, respectively. Conversely, overactivity of DRac rescued the loss of veins of Raf\textsuperscript{DN} flies. These genetic interactions are also manifested in the effects of DRac on the expression of a downstream target of the EGFR/Ras pathway (rho/ve). rho/ve expression is increased in flies with enhanced DRac signalling, and reduced in flies overexpressing DRacGAP, indicating that Rac signalling activates the Ras pathway. The rescue of the loss-of-vein and reduced proliferation phenotypes of Raf\textsuperscript{DN} flies by overactivity of DRac1 suggests that DRac1 functions downstream of Raf. The possibility of DRac1 acting in parallel with Raf to activate targets of the MAPK is unlikely since MEK activity is increased by enhancing Rac signalling, as visualised by the expanded distribution of phosphorylated-MAPK, the substrate of MEK. How can Rac signalling impinge on Raf-induced MEK activation? Any of the proteins which interact with Raf or MEK (Sternberg and Alberola-Ila, 1998) may be the target for posttranslational modification by any of the kinases acting as effectors of Rac (reviewed by Aspenström, 1999). Our results suggest that Rac activation of Ras pathway may be mediated by Pak, a serine/threonine kinase which binds to and is activated by Rac and Cdc42 (Aspenström, 1999) (Fig. 7). Mammalian Pak1 phosphorylates MEK1 on a site crucial for its in vivo binding to Raf, stabilising the binding of Raf to MEK1 and thereby enhancing Raf-mediated MEK1 activation (Frost et al., 1997). Moreover, mammalian Pak3 is able to positively regulate Raf1 activity through phosphorylation (King et al., 1998). Accordingly, rescue of the mutant phenotype of Raf\textsuperscript{DN} by overexpression of Rac might be accomplished by overactivated Pak enhancing the activity of the residual Raf present in Raf\textsuperscript{DN} flies.

Note that overexpression of DRac only caused thickening of the veins but did not induce development of ectopic veins, indicating that Rac activity is required to enhance the intensity of the Rac pathway in previously determined vein regions. This poses the question of how DRac activity is controlled. Since expression of DRac1 is generalised (our unpublished data), control of its activity should rely on the opposing effects of RacGEFs and RacGAPs. Activation of GEFs appears to depend on their subcellular localisation (Van Aelst and D’Souza-Schorey, 1997). Similarly, since most of the mammalian Rac-GAP proteins are ubiquitously distributed and contain putative protein interaction domains, it is assumed that they are regulated by protein-protein interactions, which may affect their activity or their subcellular localisation. However, expression of the human n- and β-chimaerins RacGAPs is tissue specific (Van Aelst and D’Souza-Schorey, 1997) and downregulation of human β-chimaerin in brain cells may be a factor in the development of malignant brain gliomas (Yuan et al., 1995). Hence, restricted expression of RacGAPs could be an alternative mechanism for Rac control. Patterned expression is found in the case of DRacGAP; its expression in late third instar larvae and early pupae is almost excluded from the vein regions and it accumulates at the intervein cells adjacent to the veins. The functional relevance of this distribution is stressed by the observation that expression of DRacGAP in vein territories reduced Ras signalling while reduction of DRacGAP
activity in the intervein territory expanded the domains of Ras pathway activity.

Interestingly, in the *Drosophila* wing disc, *D RacGAP* transcription is repressed by the EGFR/Ras pathway (Fig. 7). Activity of this pathway is finely tuned by it controlling the expression of its own inhibitors and activators (Wasserman and Freeman, 1998; Martin-Blanco et al., 1999; Perrimon and McMahon, 1999). Our results indicate that Ras signaling can self-stimulate through activation of the Rac pathway by repression of *D RacGAP* (Fig. 7). During vein formation, the EGFR/Ras pathway, once it has attained a certain threshold, should repress expression of *D RacGAP* in the prospective vein regions thus ensuring maximal activity of Rac and, in turn, of Ras pathways in these territories of the imaginal wing disc, which should trigger vein differentiation. In contrast, maximal expression of *D RacGAP* at the vein/intervein boundaries should locally decrease Rac and Ras signalling, and in collaboration with Notch and Dpp pathways (for a review see de Celis, 1998) help to refine the final width of the veins. Hence, this regulatory loop is another feedback mechanism modulating the activity of the EGFR/Ras pathway during *Drosophila* development.

We are most grateful to J. Modolell, J. Culi and M. J. García for their constant help and advice in the course of this work; A. Baonza, J. F. de Celis, E. Martin-Blanco and colleagues in our laboratory for constructive criticisms on the manuscript; E. Caminero for expert technical assistance, J. Pinilla for statistical analysis and A. Baonza, J. Bota, R. Fehon, M. Freeman, L. Luo, E. Martin-Blanco, M. Mlodzik, G. Rubin, E. Sánchez-Herrero, L. Zipursky and the Bloomberg Stock Center for materials and stocks. A postdoctoral fellowship from the Comunidad Autónoma de Madrid to S. S. is acknowledged. This work was supported by grants from Comunidad Autónoma de Madrid (07B/0035/97), Dirección General de Investigación Científica y Técnica (PB93-0181 and PB98-0682 to J. Modolell) and an institutional grant from Fundación Ramón Areces to the Centro de Biología Molecular Severo Ochoa.

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