The Drosophila Ral GTPase Regulates Developmental Cell Shape Changes through the Jun NH$_2$-terminal Kinase Pathway

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Abstract. The Ral GTPase is activated by RalGDS, which is one of the effector proteins for Ras. Previous studies have suggested that Ral might function to regulate the cytoskeleton; however, its in vivo function is unknown. We have identified a Drosophila homologue of Ral that is widely expressed during embryogenesis and imaginal disc development. Two mutant Drosophila Ral (DRal) proteins, DRal$^{G20V}$ and DRal$^{S25N}$, were generated and analyzed for nucleotide binding and GTPase activity. The biochemical analyses demonstrated that DRal$^{G20V}$ and DRal$^{S25N}$ act as constitutively active and dominant negative mutants, respectively. Overexpression of the wild-type DRal did not cause any visible phenotype, whereas DRal$^{G20V}$ and DRal$^{S25N}$ mutants caused defects in the development of various tissues including the cuticular surface, which is covered by parallel arrays of polarized structures such as hairs and sensory bristles. The dominant negative DRal protein caused defects in the development of hairs and bristles. These phenotypes were genetically suppressed by loss of function mutations of hemipterous and basket, encoding Drosophila Jun NH$_2$-terminal kinase (JNK) and Jun NH$_2$-terminal kinase (JNK), respectively. Expression of the constitutively active DRal protein caused defects in the process of dorsal closure during embryogenesis and inhibited the phosphorylation of JNK in cultured S2 cells. These results indicate that DRal regulates developmental cell shape changes through the JNK pathway.

Key words: bristle • dorsal closure • hair • Jun NH$_2$-terminal kinase • Ral

Ral is a member of the small GTPase superfamily and is found in two forms, RalA and RalB (reviewed by Feig et al., 1996). Like all the other small GTPases, Ral cycles between GTP-bound active and GDP-bound inactive forms. The GTP-bound form of Ral is changed to the guanosine 5'-diphosphate (GDP)-bound form by Ral GTPase-activating protein (RalGAP) (Emkey et al., 1991). The GDP-bound form of Ral is converted to the GTP-bound form by at least three guanine nucleotide exchange factors (GEFs): Ral guanine nucleotide dissociation stimulator (GDS) (Aibright et al., 1993), Ral GTPase dissociation stimulator-like (RGD) (Kikuchi et al., 1994; Murali et al., 1997), and Ral GDS-like factor (RLF) (Wolthuis et al., 1996). Interestingly, all of these RalGEFs interact with the GTP-bound form of Ras (Hofer et al., 1994; Kikuchi et al., 1994; Spaargaren and Bischoff, 1994). Moreover, stimulating cells with insulin or EGF results in increased amounts of Ral-GTP, due to the activation of Ral (Kishida et al., 1997; Wolthuis et al., 1998). In addition, RalGEFs and Ral have been implicated in Ras-induced DNA synthesis, gene expression, and oncogenic transformation (Umar et al., 1996; White et al., 1996; Miller et al., 1997; Okazaki et al., 1997; Wolthuis et al., 1997). In spite of accumulating evidence indicating that Ral and RalGEFs mediate some of the downstream signaling from activated Ras, the mechanisms by which Ral regulates cellular function remain unknown.
Recently, putative downstream targets for Ral have been identified (reviewed by Feig et al., 1996). Ral binds to phospholipase D (PLD) and is required for Ras- and Src-dependent activation of PLD (Jiang et al., 1995). The interaction between Ral and PLD is mediated by the NH$_2$-terminal region of Ral and is independent of Ral’s binding to nucleotides (Jiang et al., 1995). A nether putative effector of Ral is RalBP1, which binds to the effector domain of the GDP-bound form of Ral, but not to the GTP-bound form (Cantor et al., 1995; Juliien-Flores et al., 1995; Park and Weinberg, 1995). Interestingly, RalBP1 contains a RhoGAP domain and acts as a GAP for Cdc42 and Rac, suggesting that Ral may be involved in the regulation of Cdc42 and Rac (Cantor et al., 1995; Juliien-Flores et al., 1995; Park and Weinberg, 1995). These two GTPases are known to be involved in the regulation of the actin cytoskeleton and a signal transduction cascade including p21-activated kinase (PAK), Jun NH$_2$-terminal kinase kinase (JNK), and Jun NH$_2$-terminal kinase (JNK; reviewed by V an A elst and D’Souza-Schorey, 1997). Thus, it is possible that RalGEFs and Ral may function downstream of Ras to regulate the actin cytoskeleton and the JNK pathway. However, the role of Ral in these cellular events has not been determined.

During the development of multicellular organisms, a variety of morphologically differentiated cells are generated. Proper regulation of the cytoskeleton is essential for the precise changes in their shape. A well studied example of cell shape change is the development of hairs and bristles in Drosophila, in which the epithelial cells that secrete cuticle form hairs and bristles that point posteriorly or distally. A number of studies have shown that regulation of the cytoskeleton is required to regulate the development of these structures (Cant et al., 1994; Verheyen and Cooley, 1994; Tilney et al., 1993, 1996; Eaton et al., 1996; Hopmann et al., 1996; Turner and A dier, 1998; Wulfkuhle et al., 1998). Thus, development of Drosophila hairs and bristles is an ideal model system to study the function of Ral GTPase in cell shape changes.

In this paper, we report the identification and characterization of a Ral GTPase in Drosophila. Constitutively active and dominant negative mutants of Drosophila Ral (D Ral) were generated and used for functional characterization, both in vitro and in vivo. Our results indicate that Ral regulates developmental cell shape changes through inhibition of the JNK pathway.

**Materials and Methods**

**Cloning and Sequencing of the DRal cDNA**

D egenerate primers were designed to amplify Ras-like genes by PCR. The sequences were: GGG T G C A A (A/G) I A (A/T) C/G I A/C/G/T I G C/A/C/G/T/C/T/A/C/G/T/A/C and A/C/I T/C/T/C/T/G I C/C/A/C/G/T/G/C/A/C/G/T/J G/T/J/G/T/J/G/T/J/G/T. PolyA$^+$ mRNA was prepared from S2 cells using a Micro FastTrack kit (Invitrogen Corp.) and used as the template for synthesizing cDNA s using a first strand cDNA kit (Pharmacia Biotech, Inc.). The PCR procedure was five cycles at 94°C for 1 min, 46°C for 1 min, and 72°C for 1 min, followed by 45 cycles at 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min. PCR products were subcloned into the pT7 blue T vector (Novagen, Inc.), transformed into JM109 cells, and subjected to DNA sequencing according to standard protocols. The PCR product of DRal was $^{32}$P-labeled and used as a probe to screen an eye imaginal disc cDNA library. Five 1.2-kb cDNA s were identified that contained the entire open reading frame encoding DRal, a 261-bp 5’ untranslated region and a 354-bp 3’ untranslated region. The nucleotide sequence encompassing the open reading frame was determined by sequencing the cDNA s from both directions.

**Northern Blotting**

RNA samples were prepared from eye imaginal discs of third-instar larvae according to the method previously described by Fisher-Vize et al. (1992). Northern blotting analysis was performed using standard methods. The DRal cDNA was labeled with $^{32}$P-dCTP and used as a probe.

**In situ Hybridization to Polytenye Chromosomes**

The DRal cDNA was labeled with digoxigenin using a random-primer kit (Boehringer Mannheim Corp.) and hybridized with squashed Polytenye chromosomes, as described previously (Z uker et al., 1985). The chromosomes were incubated with alkaline phosphatase-coupled antidigoxigenin antibodies. The signal was developed according to the manufacturer’s instructions.

**Site-directed Mutagenesis and Plasmid Constructions**

The DRal cDNA in pBluescript was used as the template for site-directed mutagenesis with QuickChange™ and Chameleon KIts (Stratagene). The constitutively active DRal$^{L225H}$ mutation was created using an oligonucleotide with a base change from GGC to GTG, converting amino acid 20 from Gly to Val. The dominant negative DRal$^{I225M}$ mutation was created using an oligonucleotide with a base change from TCC to AAC, converting amino acid 25 from Ser to Asn. Mutations were confirmed by DNA sequencing. The cDNA inserts with or without mutations were excised from pBluescript and then ligated into either pGEX (Pharmacia Biotech, Inc.) for the expression of glutathione S-transferase (GST)-fusion proteins in E. chercichia coli, or into pUAST (Brand and Perrimon, 1993) for the generation of transgenic Drosophila lines and transfection of S2 cells.

**Purification of GST Fusion Proteins**

To purify GST fusion proteins (GST-DRal, GST-DRal$^{L225H}$, GST-DRal$^{I225M}$, and GST-RalGDS) from E. coli, transformed E. coli were initially grown in Luria-Bertani’s broth at 37°C to an absorbance of 0.8 (OD$_{600}$ nm), and subsequently transferred to 25°C. Isopropyl-1-β-D-thiogalactopyranoside was added to a final concentration of 100 μM and further incubation was carried out for 10 h at 25°C. The GST fusion proteins were purified from E. coli by glutathione Sepharose 4B, in accordance with the manufacturer’s instructions.

**RaLGDS Assay**

GST-DRal and GST-DRal mutants (8 pmol each) were preincubated for 10 min at 30°C in 20 μl of reaction mixture (50 mM Tris/HCl, pH 7.5, 2 μM $[^{32}$P]GTP [8,000–12,000 cpm/μmol], 5 mM MgCl$_2$, 10 mM EDTA, 1 mM DTT, and 1 mg/ml BSA). A fiter preincubation, 1 μl of 400 mM MgCl$_2$ was added. To this preincubuation mixture, 29 μl of reaction mixture (30 mM Tris/HCl, pH 7.5, 170 μM GTP, and 1 mg/ml BSA) containing GST-RalGDS (10 pmol) was added, and the mixture was further incubated for 5–30 min at 30°C. A ssays were quantified by rapid filtration on nitrocellulose filters (A lright et al., 1993).

**RaLGAP Assay**

RaLGAP was partially purified from bovine brain cytosol as described previously (H inouchi et al., 1996). GST-DRal and GST-DRal mutants (3 pmol each) were preincubated for 10 min at 30°C in 9 μl of the preincubation mixture (50 mM Tris/HCl, pH 7.5, 2 μM $[^{32}$P]GTP [8,000–12,000 cpm/μmol], 5 mM MgCl$_2$, 10 mM EDTA, 1 mM DTT, and 1 μg/ml BSA). A fiter preincubation, 1 μl of 340 mM MgCl$_2$ was added. To this preincubation mixture, 30 μl of reaction mixture (50 mM Tris/HCl, pH 7.5, 1.3 mM GTP, 0.3 mM MgCl$_2$, and 1 mg/ml BSA) containing RaLGAP (7 μg of protein) was added, and the second incubation was performed for 15 min at 30°C. A ssays were quantified by rapid filtration on nitrocellulose filters. The actual catalytic rates (Kcat) were calculated from the decrease in radioactive $[^{32}$P]GTP in the presence or absence of RaLGAP (H inagishimi et al., 1987).
Other Biochemical Assays of DRal

The $K_d$ values for GDP or GTP·S of, dissociation rate of GDP (K$^{-1}$) from, and the steady-state rate ($K_{ss}$) of GTP hydrolysis of the mutant forms of DRal were determined as described previously (Kikuchi et al., 1988; Shojo et al., 1989).

Genetics

Plasmids were injected into the embryos of w$^{1118}$,Dr/TM5, Sb P[yr$^{y^s}$, y2-3] (from the Bloomington stock center) to generate transgenic lines, as described previously (Sawamoto et al., 1994). w$^{1118}$ was used as the wild-type strain. GMR-GAL4 was provided by M. Freeman (MRC Laboratory of Molecular Biology, Cambridge, U.K.); GAL4-69B was provided by S.C. Fujita (Mitsubishi-Kasei Institute of Life Sciences, Tokyo, Japan); sca-GAL4 by T. Hosoya (National Institute of Genetics, Mishima, Japan); mtf1 and mtf2 by T. Rabe (Universität Würzburg, Würzburg, Germany); da-GAL4 by F. Matsuaki (Tokohu Univ., Sendai, Japan); RhoA$^{427}$ by M. Lodzik (EMBL, Heidelberg, Germany); cdc42$^{b}$ by R. Fehon (Duke Univ., Durham, NC); hep$^{75}$; hep$^{1}$; bsk$^{1}$; and Df(2L) rp147E by Y. Takatsu (National Institute for Basic Biology, Okazaki, Japan); actin-GAL4 and arm-GAL4 from M. Okabe (National Institute of Genetics, Mishima, Japan); pnr-GAL4 and LE-GAL4 from M. Tateno (Nagoya Univ., Nagoya, Japan); Ras2$^{164}$ by D. Yamamoto (Waseda Univ., Tokyo, Japan); D-raf$^{1}$, Raf$^{152}$, Raf$^{152}$, Dsor1$^{152}$, and Dsor1$^{152}$ by Y. Nishida (Nagoya Univ., Nagoya, Japan); and Df(3L) emc5, Df(3L) rpbl-X1, w$^{1118}$, and w$^{1118}$, Dr/ TM5, Sb P[yr$^{y^s}$, y2-3] by the Bloomington Stock Center. Fly crosses were performed at 25°C unless noted otherwise.

Histological Analyses

In situ hybridization to embryonic and larval tissues was performed as described by Tautz and Pfeifle (1989), using an antisense RNA probe encompassing the entire DRal cDNA. A sense probe was used in parallel as the control.

For scanning EM, adult flies or isolated wings were dehydrated in a graded ethanol series and dried using a critical point dryer. The mounted samples were ion-coated and observed with a scanning electron microscope (Hitachi Instruments, Inc.).

For immunohistochemical and pupal wing samples, 5 μm roughened sections from the pupal wing were dissected away from the surrounding cuticle and fixed in 8% paraformaldehyde/PBS at room temperature for 20 min. The wing samples were washed in 0.1% Triton X-100/PBS (PBT) three times, then incubated in rhodamine-phalloidin/PBT (0.5 mg/ml; Sigma Chemical Co.) overnight at 4°C. After rinsing in three changes of PBT, the wings were mounted and examined with a confocal laser scanning microscope (Olympus).

Other Biochemical Assays of DRal

The DRal mRNA was also broadly expressed in the brain hemispheres and ventral nerve cords (Fig. 3E), leg discs (Fig. 2D), eye discs (Fig. 2F), and wing discs (Fig. 2G).

Results

Cloning the cDNA that Encodes the DRal Protein

The known GTPase genes of the Ras family share significant homology in several structurally and functionally important regions. To search for novel Ras-like GTPases in Drosophila, we designed degenerative PCR primers that recognize the nucleotide binding and effector regions of the known GTPases of the Ras family and that were also likely to amplify novel Ras-like GTPases. Using these primers to perform reverse transcriptase PCR, we isolated a number of cDNAs encoding Ras-like GTPases. Some were known genes, such as Ras1 (Simon et al., 1991), Ras2 (Neuman-Silberberg et al., 1984), roughened (Harihara et al., 1991), and Rnc (Wes et al., 1996), and others represented a novel gene similar to Ral. We used a PCR fragment with a sequence homologous to Ral as a probe to screen an eye imaginal disc cDNA library and isolated a 1.2-kb cDNA clone. The size of the cDNA was similar to that of the transcript detected by Northern analysis of RNA from eye antennal discs in third-instar larvae using the same cDNA as a probe (data not shown).

The sequence of the cDNA indicated a single open reading frame encoding a protein of 201 amino acids with a predicted molecular mass of 21 kD. The deduced amino acid sequence shared high homology with all of the mammalian Ral proteins (Fig. 1). The amino acid sequence in the putative effector domain was identical to that of the mammalian Ral proteins. The CAAX motif at the COOH terminus required for geranylgeranylation (Hinoi et al., 1996) was also conserved. Based on the sequence similarity, we named the gene DRal.

To determine the cytological map position of the DRal gene, we performed in situ hybridization with chromosomes from Drosophila salivary glands using the DRal cDNA as the probe. A single signal was detected in the 3E region on the X chromosome (data not shown).

Cell Culture and Transfection Assay

pWAGAL4 was a kind gift from Dr. Y asushi Hiroi (National Institute of Genetics, Japan). S2 cells were grown on 24-well plates to 60–80% confluency in Schneider’s medium (Sigma Chemical Co.) supplemented with 10% fetal bovine serum. S2 cells were transfected with pWAGAL4 (200 ng) alone, pWAGAL4 (200 ng) plus pUA3 (200 ng) or pUA3 (200 ng) plus pUA5 (200 ng) using Cell Fectin reagent ( Gibco BRL) according to the manufacturer’s instructions. A fter 24 h, the cells were incubated in Drosophila serum-free medium ( Gibco BRL) for 30 min, then treated with 500 mM α-sorbtol for 5 min. Cells were lysed in 40 μl of SDS-PAGE sample buffer containing phosphatase inhibitors (100 μM okaidic acid, 200 μM sodium orthovanadate, and 50 μM sodium fluoride), heated at 100°C for 3 min and spun at 10,000 g for 10 min. The resulting supernatant fractions were subjected to SDS-PAGE (12.5% gel) and transferred to a nylon membrane. A filter blocking in 5% dry milk in PBS + 0.1% Tween-20 overnight at 4°C, the membranes were incubated with anti-ACTIVE JNK (Promega Corp.) or anti-JNK1 (Santa Cruz Biotechnology) antibodies for 1 h at room temperature, and then with HRP-conjugated anti-rabbit IgG (Jackson Immuno-Research Laboratories, Inc.) for 1 h at room temperature. Signals were detected by ECL reagents (Nycomed A mersham Inc.).

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Biochemical Characterization of the Constitutively Active and Dominant Negative Mutants of the Drosophila Ral Protein

Since no mutants of the Drosophila Ral gene were available, we designed constitutively active and dominant negative Drosophila Ral mutants based on structure–function studies of human Ral (Hinoi et al., 1996). Point mutations at two positions in Drosophila Ral: G20V and S25N, were generated. The Drosophila RalG20V (Val-20 for Gly-20) mutation corresponds to Ras G12V, which was originally identified in an oncogenic form of Ras and is shown to render Ras constitutively active (reviewed by Barbacid, 1996). The DRalS25N (Asn-25 for Ser-25) mutation corresponds to Ras S17N, which was originally identified by its preferential binding to GDP over GTP (Feig and Cooper, 1988). RasS17N may function as a dominant negative mutant by sequestering the GEF (Schweighoffer et al., 1993).

Previously, we characterized the biochemical activities of human wild-type Ral and its mutants (Hinoi et al., 1996). To examine the biochemical characteristics of the Drosophila Ral mutants used here, we inserted wild-type Drosophila Ral and the two Drosophila Ral mutants (DralG20V and DralS25N) into bacterial expression vectors and purified them as GST fusion proteins. The characterization of these Drosophila Ral mutants is summarized in Table I. The KD values of wild-type Drosophila Ral for GDP and GTP•γS were similar (~14 and 31 nM, respectively). DralG20V also showed similar KD values for both GDP and GTP•γS. The KD values of DralG20V for GDP and GTP•γS were larger than those of wild type, and its affinity for GDP was four- to fivefold higher than for GTP•γS. The GDP dissociation constants (Kd) of wild-type Drosophila Ral, DralG20V, and DralS25N were 0.009, 0.006, and 0.09, respectively.RalGDS stimulated the dissociation of GDP from Drosophila Ral four- to fivelfold. RalGDS stimulated the dissociation of GDP from Drosophila RalG20V threefold, but did not affect that from DralS25N. The steady-state rates (Kcat) of the GTPase activity of Drosophila Ral, DralG20V, and DralS25N were 0.007, 0.003, and 0.004, respectively. RalGDS stimulated the actual GTPase Kcat of wild-type Drosophila Ral eightfold, but not that of Drosophila RalG20V. The biochemical characteristics of Drosophila RalG20V and DralS25N were almost identical to those of human Ral G20V and Ras S28N, respectively. These results indicate that Ser-25 of Drosophila Ral is important for the action of RalGDS, that Gly-20 is important for the action of RalGAP, and that Drosophila RalG20V and Drosophila RalS25N are constitutively active and dominant negative forms of Drosophila Ral, respectively.

Table I. Kinetic Parameters of Drosophila Ral

|                  | GDP dissociation constant Kd (nM) | GTPase (actual catalytic rate) Kcs (1,000 (min⁻¹)) |
|------------------|-----------------------------------|-----------------------------------------------|
|                  | GDP                               | GAP                                           |
|                  | GTP•γS                            | +GAP                                         |
| Wild-type        | 14.0                              | 30.7                                          |
|                  | 8.6                               | 39.6                                          |
|                  | 4.6                               |                                               |
| DralG20V         | 8.4                               | 38.0                                          |
|                  | 5.8                               | 17.9                                          |
|                  | 3.1                               |                                               |
| DralS25N         | 95.1                              | 406.0                                         |
|                  | 86.6                              | 86.6                                          |
|                  | 1.0                               |                                               |

To determine the KD values of Dral mutants for the guanine nucleotides, Dral mutants (1 pmol) were incubated for various periods of time at 30°C with various concentrations of [3H]GDP or [35S]GTP•γS in 100 μl of reaction mixture (50 mM Tris/ HCl, pH 7.5, 5 mM MgCl2, 1 mM DTT, and 1 mg/ml BSA; Kikuchi et al., 1988). To determine the RalGDS activity for Dral mutants, the [3H]GDP-bound form of Dral mutants (8 pmol) were incubated with or without 200 nM RalGDS for various periods of time at 30°C. Kd for GTP•γS was determined as described (Shoji et al., 1989). Kcs of the GTPase activity was determined by incubating Dral mutants with [γ-32P]GTP for various periods of time at 30°C and expressed as the turnover number (Kikuchi et al., 1988). Kcs of GTPase activity was determined in the presence or absence of RalGAP (7 μg of protein) as described by Hidajatulima et al. (1987). Kcs for DralG20V was assayed at 5 μM GDP instead of 1 μM GDP, which was employed for the assay of wild-type Drosophila Ral and DralS25N. The Kcs of DralS25N was determined because most of the γ-32PGTP was dissociated during these assays. The results shown are the means of three independent experiments.
Functional Analysis of DRal by Ectopic Expression

To gain insight into the function of DRal in Drosophila development, we examined the effects of overexpressing the dominant mutants described in a specific tissue using the GAL4/UAS (upstream activation sequence) system (Brand and Perrimon, 1993). The cDNAs encoding the wild-type, constitutively active (G20V), and dominant negative (S25N) DRal proteins were subcloned into the transformation vector pUAST (Brand and Perrimon, 1993), and used to generate transgenic lines. The pUAST vector contains the UAS that is responsive to the yeast transcription factor GAL4. We then crossed these transgenic flies to several GAL4 lines.

For all of the experiments in this study, at least two independent UAS-DRal lines were examined and found to show similar phenotypes.

Overexpression of the wild-type DRal protein did not cause any visible phenotype. On the other hand, overexpression of DRalG20V and DRalS25N resulted in a variety of phenotypes that depended on the GAL4 line used. In this study, we focused on the effect of DRalS25N on the development of two cell types that have highly specialized structures, hair and bristles, since the phenotypes were obvious and easy to analyze. The development of these structures is dependent on the proper regulation of the cytoskeleton (Adler, 1992; Wulfkuhle et al., 1998).

Effects on Wing Hair

Each epithelial cell of the Drosophila wing forms a hair by extending a single process from its apical membrane during pupal development (Mitchell et al., 1983; A dler, 1992; Fristrom et al., 1993; W one and A dler, 1993). A t ~35 h after puparium formation (A PF), F-actin accumulates on the distal side of the epithelial cells. Subsequently, outgrowth of prehairs is initiated from the distal side (Wone and Adler, 1993). To examine if DRal is involved in hair outgrowth, wild-type and dominant negative DRal proteins were misexpressed in developing wings, using the GAL4 line 69B (Brand and Perrimon, 1993). To observe the fine structure of the hair, wing samples were examined with a scanning electron microscope (Fig. 3, A–E). The wild-type wing hairs were evenly spaced with distal polar-
ity (Fig. 3 A). The hairs on the wings overexpressing the wild-type DRA1 protein were morphologically indistinguishable from the wild-type hair (data not shown). On the wings expressing DRA1S25N, the cells often formed multiple wing hairs (Fig. 3, B, D, and E). The hairs were also shorter than in wild type (Fig. 3 B). Moreover, some hairs were forked, curved, or twisted (Fig. 3, B and D). The abnormal appearance of the hairs suggests that the organization of the actin cytoskeleton in the hairs may have been defective.

To label the F-actin, the developing wings were dissected from pupae at 30-36 h APF and stained with rhodamine-conjugated phalloidin. In the wild-type pupal wings, a single large bundle of F-actin, termed the prehair, is formed in each wing cell (Wong and Adler, 1993; Fig. 3 F). In the developing wings expressing DRA1S25N, cells with two or three prehairs were occasionally seen (Fig. 3 G). In addition, the morphology of the prehairs was irregular (Fig. 3 G). These data suggest that DRA1 is required for regulation of the initiation process during hair development.

Effects on Sensory Bristles

The development of sensory bristles provides another excellent model system to study how the cytoskeleton controls cell shape changes. Each external sense organ consists of four cells: the neuron, the sheath, the tormogen (socket forming cell), and the trichogen (shaft forming cell; Hartenstein and Posakony, 1989). During pupal development, a cytoplasmic extension of the trichogen becomes the bristle shaft. To induce expression of the DRA1 proteins in the developing trichogens, UAS-DRA1 flies were crossed to the sca-GAL4 line. The bristles of flies expressing the wild-type DRA1 protein were indistinguishable from those of wild-type (Fig. 4, A and C): their length, morphology, and orientation were normal (data not shown). On the other hand, the expression of DRA1S25N resulted in the loss of bristles on the nota (Fig. 4, B and D). In some cases, DRA1S25N affected both shafts and sockets, suggesting that DRA1 may be involved in the development of these structures. Both macrochaetes (large bristles) and microchaetes (small bristles) were affected by the DRA1S25N expression.

The absence of bristles on the nota expressing DRA1S25N could be due to failure in the process of shaft initiation from the trichogen cells. Alternatively, overexpression of the dominant negative DRA1 protein could disrupt the formation of the trichogen cells. To distinguish between these two possibilities, developing nota from pupae at 26-32 h APF were stained with the antibody mAb 22C10 (Fujita et al., 1982). At this stage, mAb 22C10 labeled at least two cells in each macrochaete and microchaete on the wild-type nota: a neuron sending out axons and a trichogen cell. Developing shafts contained F-actin were observed at this stage. On the nota expressing DRA1S25N, initiation of shafts was often inhibited (Fig. 6 B). Fig. 6 C shows a wild-type macrochaete. The developing shaft was filled with well-organized actin bundles that ran parallel to the long axis of the bristle. At the tip, patches of F-actin were observed. On the nota expressing DRA1S25N, the development of actin structures in the macrochaetes appeared to be interrupted at the initiation of extension (Fig. 6 D).

Next, we used the GAL4-69B line to examine the effects of expressing wild-type DRA1 and DRA1S25N in the trichogen and hair cells on the nota. The phenotype of the hairs on the nota was similar to that of the wing hairs (Fig. 3). They were often shortened, forked, twisted, duplicated, or triplicated (Fig. 7, A and C). As for the bristles, the GAL4-69B line expressing DRA1S25N resulted in a similar phenotype to that caused by sca-GAL4 (i.e., some of the bristles were lost; Fig. 7, A and C). We expected that these phenotypes were caused by a dominant negative effect on the endogenous DRA1 protein. To address whether these phenotypes could be caused by decreased function of DRA1, wild-type DRA1 protein was expressed with the dominant negative DRA1S25N protein. The loss of bristles and morphological defects resulting from DRA1S25N expression were largely rescued by coexpression of the wild-type DRA1 protein (Fig. 7, B and D). Therefore, these ple-
nototypes are likely to have resulted from decreased DRal function.

Effects of Mutations in the JNK Pathway on the DRalS25N-induced Phenotype

To explore other genes associated with the DRal-induced defects described above, flies carrying both sca-GAL4 and UAS-DRalS25N were crossed to a number of mutants for genes known to be involved in the Ras pathway and cytoskeletal regulation. The resulting F1 progenies were scored for modification of the bristle-loss phenotype caused by DRalS25N (Table II). No effect was seen as a result of halving the dosages of the genes coding for the proteins of the Ras/Raf/ERK pathway or the Rho family of small GTPases, i.e., Ras1 (Simon et al., 1991), D-raf (Nishida et al., 1988), Dsor1 (encoding an MEK; Tsuda et al., 1993), rolled (encoding an ERK; B runner et al., 1994), mbt (encoding a PAK; Melzig et al., 1998), RhoA (Hariharan et al., 1995; Strutt et al., 1997), DCdc42 (Luo et al., 1994; Fehon et al., 1997), DRac1 (Luo et al., 1994), and DRac2 (Luo et al., 1994; data not shown). However, we found that mutations of the genes encoding JNK and JNK dominantly suppressed the DRalS25N-induced bristle phenotype (Fig. 8). Two alleles of the hemipterous (hep) gene (encoding a JNK; Glise et al., 1995) and three alleles of the bas-

Figure 5. Examination of cell types in microchaetes and macrochaetes by labeling with mAb 22C10. Staining was performed on whole-mount preparations of pupal nota at 26–32 h A PF. At the terminal stages of development shown here, the mAb 22C10 (Fujita et al., 1982) stained the cytoplasm of the neuron (n) and axons (arrowheads), and the shaft cell (s) and developing bristles (arrows). (A) Wild-type microchaete. (B) Microchaete from a DRalS25N-expressing cell. The arrow in the inset points to a malformed bristle. (C) Wild-type macrochaete. (D) Macrochaete from a DRalS25N-expressing cell. Flies analyzed were reared at 29°C.

Figure 6. Bristles from 26–32 h A PF pupae stained with rhodamine-conjugated phalloidin. (A) Wild-type microchaete. Developing bristles filled with F-actin are marked by arrowheads. (B) Macrochaete from a DRalS25N-expressing cell. A bristle that failed to grow can be identified by the accumulation of F-actin. (C) Wild-type macrochaete. An arrow points to a growing bristle that contains a number of actin bundles. (D) Microchaete from a DRalS25N-expressing cell. An arrow points to the site where F-actin is accumulated, but not extended as bristle. Flies analyzed were reared at 29°C.
Table II. Dominant Effects of Mutations of the Genes Involved in Intracellular Signal Transduction on the Bristle Phenotype Caused by DRalS25N

| Mutant alleles tested | Effects |
|-----------------------|---------|
| mbrP1                 | No effect |
| mbrP2                 | No effect |
| cdsM2                 | No effect |
| Df(3L)lenc5 (Drac1+)   | No effect |
| Df(3L)lphl-X1 (Drac2+) | No effect |
| RhoA09236             | No effect |
| RhoA09003             | No effect |
| RhoA09236             | No effect |
| hep-75                | Suppression |
| bskP447E              | Suppression |
| bskP471               | Suppression |
| bsk                 | Suppression |
| Ras1F28               | No effect |
| Draf1                 | No effect |
| DorsalP158            | No effect |
| DorsalP158            | No effect |
|メ p53664              | No effect |
| p5523                 | No effect |

w1118/Y; sca-GAL4/sca-GAL4; UAS-DRal825N/MKRS; Sh males were crossed to females of the indicated mutant stocks. The resulting F1 progenies carrying the mutation indicated, and sca-GAL4 and UAS-DRal825N were compared with the flies carrying sca-GAL4 and UAS-DRal825N, but no mutation. Flies analyzed were reared at 29°C.

It has been shown that both the bsk and hep mutations disrupt the process of dorsal closure during embryonic development (Gidée et al., 1995; Riesgo-Escovar et al., 1996; Sluss et al., 1996). Dorsal closure is a morphogenetic process in which the two sheets of lateral epidermis are elongated along their dorsoventral axes. On meeting at the dorsal midline, the two leading edges suture. If DRal functions to downregulate the JNK pathway, the constitutively active DRal protein should affect the process of dorsal closure. To induce the expression of DRalG20V in the embryonic epidermis, UAS-DRalG20V lines were crossed to a number of GAL4 lines such as actin-GAL4, 69B-GAL4, and arm-GAL4. Embryonic expression of DRalG20V resulted in lethality (data not shown). In some cases, the cuticle of the embryos showed defects on the dorsal surface (data not shown), indicating that dorsal closure was defective. To test whether expression of activated DRal in the leading edge is sufficient to induce the dorsal cuticle phenotype autonomously, DRalG20V was expressed using prn-GAL4 (Fig. 9) and LE-GAL4 (data not shown), which target expression specifically to the leading edge. The cuticle patterns in these GAL4 lines were normal and indistinguishable from those of the wild-type embryos (data not shown). Expression of DRalG20V in the leading edge specifically caused the appearance of large holes in the anterior or dorsal epidermis (Fig. 9, A and B). Some embryos showed a severe dorsal-open phenotype (Fig. 9 C) similar to the phenotypes caused by bsk and hep mutations (Gliese et al., 1995; Riesgo-Escovar et al., 1996; Sluss et al., 1996). These results suggest that activated DRal inhibited the activation of JNK in the leading edge.

DRal Inhibits the Phosphorylation of JNK in Cultured Cells

The genetic data suggested that DRal could act as a negative regulator of the JNK pathway in vivo. We next examined the ability of the constitutively active DRal protein to inhibit JNK activation when overexpressed in tissue culture cells. JNK is activated by phosphorylation on both threonine and tyrosine residues in the Thr-X-Tyr sequence within the catalytic core of the enzyme. Therefore, the level of Bsk/JNK activation in cells was evaluated on Western blots using an antibody that specifically recognizes phosphorylated JNK. S2 cells were transfected with pUAST-DRal or pUAST-DRalG20V together with a plasmid that expresses GAL4 under control of the actin5C promoter, pWAGAL4 (Hiromi, Y., unpublished observa-
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Figure 8. Mutations of the genes encoding JNKK and JNK suppress the DRalS25N-induced bristle phenotype. (A) +/+; sca-GAL4/Cyo; UAS-DRalS25N/+ (control). (B) hepS75/+; sca-GAL4/Cyo; UAS-DRalS25N/+ (hepS75). (C) +/+; sca-GAL4/bskS25N; UAS-DRalS25N/+ (bskS25N). The bristle-missing phenotype caused by expression of DRalS25N (A) was dominantly suppressed by the loss of function mutations hepS75 (B) and bskS25N (C). For other genotypes, see Table II. Flies analyzed were reared at 29°C.

Discussion

We have identified a Drosophila gene, DRal, that encodes a protein with strong homology to mammalian Ral GTPases. The Ral proteins identified in mammals so far are easily classified into two types, RalA and RalB, based on their amino acid sequence. Although the amino acid sequence of DRal is more homologous to that of RalA, some residues of DRal, e.g., Glu-103 and Pro-135, are identical to RalB, but not to RalA. Therefore, we could not classify DRal as a homologue of either RalA or RalB. The COOH-terminal region of DRal contains a basic amino acid repeat and a CAAX motif, which are important for post-translational modifications and membrane localization. DRal may be localized to the membrane with Ras and activated by RalGDS, as shown in mammals (Hinoi et al., 1996). The sequences of the effector domains of the Drosophila (from Tyr-40 to Tyr-48) and mammalian Rals are identical, suggesting that the target molecules of Ral are also conserved. There are four domains conserved in all the small GTPases, called I, II, III, and IV. I and II are important for GTPase activity, whereas II, III, and IV are important for nucleotide binding. The sequence of DRal in domains I (from Gly-18 to Lys-24), II (from Asp-65 to Gly-68), III (from Asn-124 to Asp-127), and IV (from Glu-152 to Lys-156) are well conserved with those of mammalian Rals. The structural similarity suggests that DRal is biochemically similar to the mammalian Rals. In fact, DRal bound to GTP and GDP with high affinities and showed a low intrinsic GTPase activity. DRal responded well to mammalian RalGDS and RalGAP. Moreover, a GTPase-deficient protein that is constitutively active could be made by introducing a mutation found in human Ral (Hinoi et al., 1996). Likewise, a dominant negative mutant that displays preferential affinity for GDP could be generated by introducing the mutation at the same position as in human Ral (Hinoi et al., 1996).

Much of our knowledge about the functions of small GTPases has been obtained from studies using dominant active and dominant negative mutants. In Drosophila, ectopic expression of wild-type or mutant proteins has been successfully used to study the roles of small GTPases in development (see Luo et al., 1994; Harden et al., 1995; Harbharan et al., 1995; Eaton et al., 1996; Barrett et al., 1997; Strutt et al., 1997; Hacker and Perrimon, 1998). Since no mutant flies exist that affect DRal function at present, we have used overexpression of a dominant negative protein to investigate the biological function of DRal. The advantage of this approach is that we can control the effect of the DRal mutation spatiotemporally using the GAL4/UAS system (Brand and Perrimon, 1993). The substitution of asparagine for glycine at amino acid 17 in Ral as inhibits GTP binding and sequesters the GEFs from the endogenous Ral protein (Farnsworth and Feig, 1991). Therefore, the DRalS25N protein may also function to sequester RalGDS, thereby inhibiting the activation of the endogenous DRal protein, although a RalGDS-like protein has not been identified in Drosophila. The DRalS25N-induced phenotype reported in this paper is likely to be due to a reduction in the activity of the endogenous DRal protein, because the phenotype is rescued by coexpression of the wild-type DRal protein.

Development of wing hairs is controlled by both actin and microtubules (Turner and Adler, 1998). A number of genes involved in wing hair formation have been identified. For example, wing cells of mutants for the tissue po-
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larity genes such as frizzled, disheveled, prickle, fuzzy, and multiple wing hair extend more than one prehair (Wong and Adler, 1993). These genes may play an important role in restricting the initiation site for hair outgrowth. Expression of DRalS25N also resulted in the extension of multiple prehairs from a single cell (Fig. 3). Therefore, it is possible that DRal functions to regulate prehair initiation. Moreover, close examination of the hairs with a scanning electron microscope revealed that the expression of DRalS25N affected their structure. Wing cells that expressed DRalS25N produced hairs that were deformed and stunted. We conclude that DRal plays essential roles in both the initiation of hair outgrowth and hair extension.

Another structure examined in this work is the external sensory bristle. The development of bristles is also an excellent model system for studying the role of the cytoskeleton in cell shape changes. The trichogen cell extends and forms a bristle shaft during early pupal development (Hartenstein and Posakony, 1989). This cytoplasmic extension of the trichogen cell contains a central core of microtubules surrounded by F-actin bundles (Overton, 1967; Appel et al., 1993; Tilney et al., 1996). Mutations in the genes encoding actin binding proteins result in aberrant bristle formation, suggesting that the actin cytoskeleton plays an important role in bristle development (Cant et al., 1994; Petersen et al., 1994; Verheyen and Cooley, 1994). Microtubules also have roles in bristle development (Turner and Adler, 1998). DRal may regulate the cytoskeleton organization in developing bristles, since the dominant negative DRal protein inhibited the initiation of bristles.

Our genetic and biochemical data suggest that DRal regulates cell shape changes through the inhibition of the JNK pathway (Figs. 8, 9, and 10; Table II). The JNK pathway has been implicated in cell shape changes and in the regulation of tissue polarity (for review see Noselli, 1998). The precise mechanisms for the regulation of JNK signaling by DRal are unknown. However, identification of RalBP1 as a putative effector protein of mammalian Ral may provide a clue to the mechanism (Cant et al., 1995; Jullien-Flores et al., 1995; Park and Weinberg, 1995). RalBP1 acts as a GAP for CDC42 and Rac (Cant et al., 1995; Jullien-Flores et al., 1995; Park and Weinberg, 1995).

In mammalian cells, CDC42 and Rac upregulate the JNK pathways via PAK (Coso et al., 1995). Similarly, DCdc42 and DRac1 are upstream activators of Hep/JNKK and Bsk/JNK in Drosophila (Glise and Noselli, 1997). The Drosophila homologues of PAK, DPAK and Mbt, may transduce the signal from DRac1 to the JNK pathway (Harden et al., 1996; Melzig et al., 1998). We have shown that the DRalS25N-induced phenotype could be suppressed by halving the dosages of Hep/JNKK and Bsk/JNK. Experiments were also performed to determine if the DRalS25N phenotype could be rescued by expressing a constitutively active form of DRal (G20V). In embryos expressing DRalG20V, the expression of DRal did not affect the amount of the Bsk protein. Right, phosphorylation of Bsk was detected using an antibody that specifically recognizes phosphorylated JNK. Expression of DRalG20V did not affect the amount of the Bsk protein. Right, phosphorylation of Bsk was detected using an antibody that specifically recognizes phosphorylated JNK. DRalG20V showed an inhibitory effect on the phosphorylation of Bsk. Samples were analyzed from nine culture dishes and similar results were obtained.
pression of constitutively active DRalG20V in the leading edge caused dorsal closure defects similar to those seen in JNK pathway mutants, supporting our idea that activation of DRal leads to downregulation of the JNK pathway. We also provided biochemical evidence showing that DRal could act as an upstream negative regulator of JNK activation. Consistently, the dorsal open phenotype of the bsk null mutants was not affected by expression of the dominant negative and constitutively active DRal mutants (data not shown). It is possible that DRal activates a GAP for the Cdc42 and Rac families of GTPases, resulting in a negative effect on the JNK signaling. It has recently been reported that Ral-GEFs suppress the neurite outgrowth of PC12 cells through inhibition of Cdc42 and Rac (Goi et al., 1999). However, we could not detect any modifying effect of the mutations of D Cdc42, DRac1, DRac2, and RhoA on the DRalG20V-induced phenotype. One explanation for this result is that the multiple GTPases of the Rho family may have redundant functions for activating the JNK pathway. Alternatively, DRal may negatively regulate the JNK pathway independently of the known members of the Rho family.

Ras mediates its diverse biological functions by activating multiple downstream targets including GEFs for Ras (Vojtek and Der, 1998). Ras mediates its effects on cellular proliferation in part by activating Raf (reviewed by Bos, 1993; Vojtek and Der, 1998). Ras is also known to have effects on the cytoskeleton (Bar-Sagi and Feramisco, 1986; Ridley and Hail, 1992). Rodriguez-Viciana et al. (1997) have reported that activation of the phosphoinositide 3-kinase, one of the Ras effectors, is essential in Ras-induced cytoskeletal rearrangement. Our data suggest that Ras, which is activated by another family of Ras effectors, the Ral-GEFs, also regulates the cytoskeleton through the JNK pathway, and thus plays a role in the cell shape changes that occur in animal development.

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