The *Drosophila* Ral GTPase Regulates Developmental Cell Shape Changes through the Jun NH_2-terminal Kinase Pathway

Kazunobu Sawamoto,* Per Winge,‡ Shinya Koyama,§ Yuki Hirota,* Chiharu Yamada,* Sachiyo Miyao,* Shingo Yoshikawa,‖ Ming-hao Jin,*¶ Akira Kikuchi,§ and Hideyuki Okano*¶

*Division of Neuroanatomy, Department of Neuroscience, Biomedical Research Center, Osaka University Graduate School of Medicine, Osaka 565-0871, Japan; ‡Unigen Center for Molecular Biology, Norwegian University of Science and Technology, Trondheim N-7005, Norway; §Department of Biochemistry, University of Hiroshima School of Medicine, Hiroshima 734-8551, Japan; ‖Department of Molecular Neurobiology, Institute of Basic Medical Sciences, University of Tsukuba, Ibaraki 305-0006, Japan; and ¶CREST, Japan Science and Technology Corporation at Division of Neuroanatomy, Department of Neuroscience, Biomedical Research Center, Osaka University Graduate School of Medicine, Osaka 565-0871, Japan

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 kinase (JNKK) 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)[1] (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) (Albright et al., 1993), Ral GDP dissociation stimulator-like (RGL) (Kikuchi et al., 1994; Murai 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 Ras (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 (Urano 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 RalGDS mediate some of the downstream signaling from activated Ras, the mechanisms by which Ral regulates cellular function remain unknown.

Address correspondence to Hideyuki Okano, Division of Neuroanatomy (D12), Department of Neuroscience, Biomedical Research Center, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan. Tel.: 81-6-6879-3581. Fax: 81-6-6879-3589. E-mail: okano@nana.med.osaka-u.ac.jp

1. *Abbreviations used in this paper:* APF, after puparium formation; *bsk*, *basket* gene; DRal, *Drosophila* Ral; GAP, GTPase-activating protein; GDP, guanosine 5′-diphosphate; GDS, guanine nucleotide dissociation stimulator; GEFs, guanine nucleotide exchange factors; GST, glutathione S-transferase; *hep*, *hemipterous* gene; JNK, Jun NH_2-terminal kinase; JNKK, Jun NH_2-terminal kinase kinase; PAK, p21 activated kinase; PLD, phospholipase D; UAS, upstream activation sequence.

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). Another putative effector of Ral is RalBP1, which binds to the effector domain of the GTP-bound form of Ral, but not to the GDP-bound form (Cantor et al., 1995; Jullien-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; Jullien-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 (JNKK), and Jun NH_2-terminal kinase (JNK; reviewed by Van Aelst 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., 1995, 1996; Eaton et al., 1996; Hopmann et al., 1996; Turner and Adler, 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 (DRal) 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

Degenerate primers were designed to amplify Ras-like genes by PCR. The sequences were: GGIGTIGGIAA(A/G)(A/T)(C/G)(A/C/G/T)GC(A/C/G/T)(C/T)T(A/C/G/T)AC and A(C/T)TC(C/T)TGICC(A/C/G/T)GC(A/C/G/T)GT(A/G)TC. PolyA$^+$ mRNA was prepared from S2 cells using a Micro Fast Track kit (Invitrogen Corp.) and used as the template for synthesizing cDNAs 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 cDNAs 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 cDNAs 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 Polytene Chromosomes

The *DRal* cDNA was labeled with digoxigenin using a random-primer kit (Boehringer Mannheim Corp.) and hybridized with squashed Polytene chromosomes, as described previously (Zuker 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^{G20V} mutation was created using an oligonucleotide with a base change from GGC to GTC, converting amino acid 20 from Gly to Val. The dominant negative DRal^{S25N} 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 *Escherichia 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^{G20V}, GST-DRal^{S25N}, 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 [^{3}H]GDP [1,500–3,000 dpm/pmol], 5 mM $MgCl_2$, 10 mM EDTA, 1 mM DTT, and 1 mg/ml BSA). After preincubation, 1 μl of 400 mM $MgCl_2$ was added. To this preincubation mixture, 29 μl of reaction mixture (50 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. Assays were quantified by rapid filtration on nitrocellulose filters (Albright et al., 1993).

RalGAP Assay

RalGAP was partially purified from bovine brain cytosol as described previously (Hinoi 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 γ[^{32}P]GTP [8,000–12,000 cpm/pmol], 5 mM $MgCl_2$, 10 mM EDTA, 1 mM DTT, and 1 μg/ml BSA). After 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. Assays were quantified by rapid filtration on nitrocellulose filters. The actual catalytic rates (K_{cat}) were calculated from the decrease in radioactive γ[^{32}P]GTP in the presence or absence of RalGAP (Higashijima et al., 1987).

Other Biochemical Assays of DRal

The K_d values for GDP or GTPγS 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; Shoji et al., 1989).

Genetics

Plasmids were injected into the embryos of w^{1118}; *Dr/TMS, Sb* P[ry^+, *Δ2-3*] (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, UK); *GAL4-69B* by R. Ueda (Mitsubishi-Kasei Institute of Life Sciences, Tokyo, Japan); *sca-GAL4* by T. Hosoya (National Institute of Genetics, Mishima, Japan); mbt^{P1} and mbt^{P2} by T. Raabe (Universitaet Wuerzburg, Wuerzburg, Germany); *da-GAL4* by F. Matsuzaki (Tohoku Univ., Sendai, Japan); $RhoA^{P2}$ by M. Mlodzik (EMBL, Heidelberg, Germany); $cdc42^1$ by R. Fehon (Duke Univ., Durham, NC); hep^{r75}, hep^1, bsk^1, and *Df(2L)flp 147E* 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); $Ras1^{e2F}$ by D. Yamamoto (Waseda Univ., Tokyo, Japan); $D\text{-}raf^1$, rl^{Su23}, rl^{EMS64}, $Dsor1^{Gp158}$, and $Dsor1^{Su1}$ by Y. Nishida (Nagoya Univ., Nagoya, Japan); and *Df(3L)emc5*, *Df(3L)pbl-X1*, w^{1118}, and w^{1118}; *Dr/TMS, Sb* P[ry^+, *D2-3*] 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 drier. The mounted samples were ion-coated and observed with a scanning electron microscope (Hitachi Instruments, Inc.).

For phalloidin staining, pupal wings 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 microscope (Olympus).

Pupal nota were dissected and fixed in 4% paraformaldehyde/PBS as described previously (Tilney et al., 1996). The nota were stained with rhodamine-phalloidin following the same protocol used for wing samples described above. For immunohistochemistry, the fixed and washed nota were incubated in 10% normal goat serum/PBT for 30 min at room temperature and then in 1:5 mAb 22C10 (obtained from S.C. Fujita, Mitsubishi Kasei Institute of Life Sciences, Tokyo, Japan) diluted in 10% normal goat serum/PBT overnight at 4°C. After rinsing in three changes of PBT, the nota were incubated in biotin-conjugated anti-mouse IgG at a dilution of 1:200 in 10% normal goat serum/PBT for 2 h at room temperature. The signal was developed using an ABC Elite Kit (Nycomed Amersham Inc.).

Preparation and analysis of embryonic cuticle were performed as described previously (Wieschaus and Nüsslein-Volhard, 1986).

Cell Culture and Transfection Assay

pWAGAL4 was a kind gift from Dr. Yasushi Hiromi (National Institute of Genetics, Japan). S2 cells were grown on 24-well plates to 60–80% confluence in Schneider's medium (Sigma Chemical Co.) supplemented with 10% FBS and 0.5% peptone (Difco Laboratories Inc.). The cells were transfected with *pWAGAL4* (200 ng) alone, *pWAGAL4* (200 ng) plus *pUAST-DRal* (1 μg), or *pWAGAL4* (200 ng) plus $pUAST\text{-}DRal^{G20V}$ (1 μg) using Cell Fectin reagent (GIBCO BRL) according to the manufacturer's instructions. After 24 h, the cells were incubated in *Drosophila* serum-free medium (GIBCO BRL) for 30 min, then treated with 500 mM D-sorbitol for 5 min. Cells were lysed in 40 μl of SDS-PAGE sample buffer containing phosphatase inhibitors (100 nM okadic acid, 200 μM sodium orthovanadate, and 50 mM 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. After 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 ImmunoResearch Laboratories, Inc.) for 1 h at room temperature. Signals were detected by ECL reagents (Nycomed Amersham Inc.).

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* (Hariharan et al., 1991), and *Ric* (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).

DRal Expression Pattern during Development

To examine the spatiotemporal expression pattern of the *DRal* mRNA during development, in situ hybridization analysis was performed at various stages of development using a *DRal* antisense RNA probe. Widespread expression of the *DRal* transcripts was detected throughout embryogenesis (Fig. 2, A–C). In the third-instar larval stages, *DRal* mRNA was also broadly expressed in the brain hemispheres and ventral nerve cords (Fig. 2 D), leg discs (Fig. 2 E), eye discs (Fig. 2 F), and wing discs (Fig. 2 G). The sense control probe did not hybridize to these tissues (data not shown).

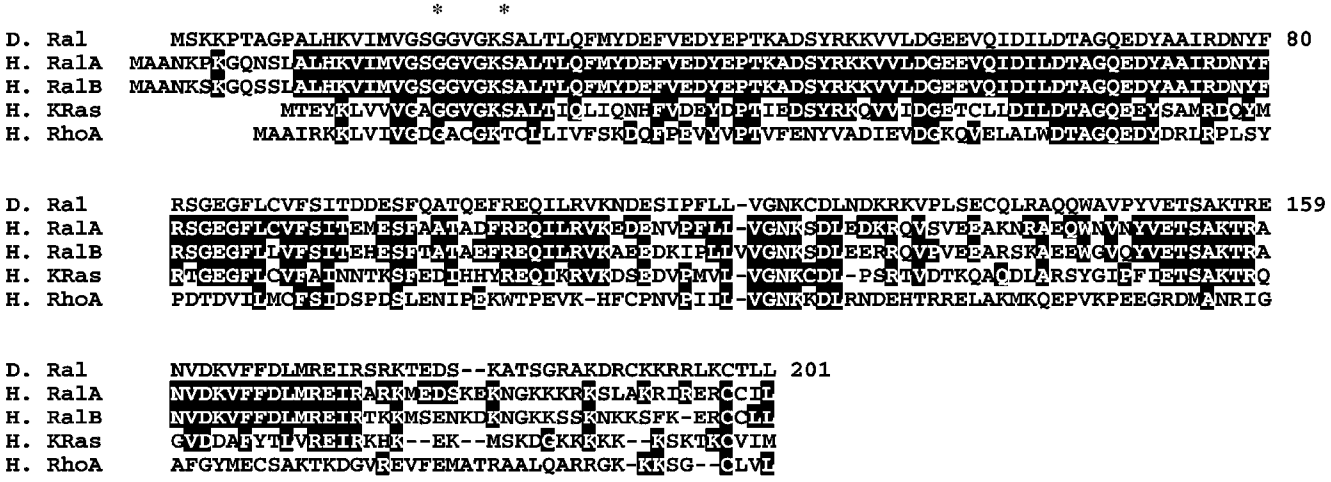

Figure 1. The amino acid sequence of *Drosophila* Ral (D. Ral) compared with human RalA (H. RalA; Chardin and Tavitian, 1989), RalB (H. RalB; Chardin and Tavitian, 1989), K-Ras (H. KRas; Kahn et al., 1987), and RhoA (H. RhoA; Moscow et al., 1994). Identities are highlighted with a black background. Gly at 20 and Ser at 25, which were mutated to Val and Asn, respectively, are indicated by asterisks. The sequence of DRal has been deposited in GenBank/EMBL/DDBJ under the accession number U23800.

Biochemical Characterization of the Constitutively Active and Dominant Negative Mutants of the DRal Protein

Since no mutants of the *DRal* gene were available, we designed constitutively active and dominant negative DRal mutants based on structure–function studies of human Ral (Hinoi et al., 1996). Point mutations at two positions in DRal, G20V and S25N, were generated. The DRal^{G20V} (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 as a result of defective GTPase activity (reviewed by Barbacid, 1987). The DRal^{S25N} (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). Ras^{S17N} 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 DRal mutants used here, we inserted wild-type DRal and the two DRal mutants (DRal^{G20V} and DRal^{S25N}) into bacterial expression vectors and purified them as GST fusion proteins. The characterization of these DRal mutants is summarized in Table I. The K_d values of wild-type DRal for GDP and GTPγS were similar (~14 and 31 nM, respectively). DRal^{G20V} also showed similar K_d values for both GDP and GTPγS. The K_d values of DRal^{S25N} for GDP and GTPγS were larger than those of wild type, and its affinity for GDP was four- to fivefold higher than for GTPγS. The GDP dissociation constants (K^{-1}) of wild-type DRal, DRal^{G20V}, and DRal^{S25N} were 0.009, 0.006, and 0.09, respectively. RalGDS stimulated the dissociation of GDP from DRal four- to fivefold. RalGDS stimulated the dissociation of GDP from DRal^{G20V} threefold, but did not affect that from DRal^{S25N}. The steady-state rates (K_{ss}) of the GTPase activity of DRal, DRal^{G20V}, and DRal^{S25N} were 0.007, 0.003, and 0.004, respectively. RalGAP stimulated the actual GTPase K_{cat} of wild-type DRal eightfold, but not that of DRal^{G20V}. The biochemical characteristics of DRal^{G20V} and DRal^{S25N} were almost identical to those of human Ral^{G23V} and Ral^{S28N}, respectively. These results indicate that Ser-25 of DRal is important for the action of RalGDS, that Gly-20 is important for the action of RalGAP, and that DRal^{G20V} and DRal^{S25N} are constitutively active and dominant negative forms of DRal, respectively.

Table I. Kinetic Parameters of Drosophila Ral

	K_d		GDP dissociation constant $K^{-1} \times 1{,}000$ (min^{-1})		
	GDP	GTPγS	−GDS	+GDS	GDS sensitivity
	nM				*-fold*
Wild-type	14.0	30.7	8.6	39.6	4.6
Ral^{G20V}	8.4	38.0	5.8	17.9	3.1
Ral^{S25N}	95.1	406.0	86.6	86.6	1.0

GTPase (steady-state rate) $K_{ss} \times 1{,}000$ (min^{-1})	GTPase (actual catalytic rate) $K_{cat} \times 1{,}000$ (min^{-1})		
	−GAP	+GAP	GAP sensitivity
			-fold
7.1	23.1	187.3	8.1
3.4	21.1	22.7	1.1
4.3	ND	ND	ND

To determine the K_d 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 [$^{-3}$H]GDP or [^{35}S]GTPγS in 100 μl of reaction mixture (50 mM Tris/HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, and 1 mg/ml BSA; Kikuchi et al., 1988). To determine the RalGDS activity for DRal mutants, the [$^{-3}$H]GDP-bound form of DRal mutants (8 pmol) were incubated with or without 200 nM RalGDS for various periods of time at 30°C. K^{-1} was determined as described (Shoji et al., 1989). K_{ss} of the GTPase activity was determined by incubating DRal mutants with γ[^{32}P]GTP for various periods of time at 30°C and expressed as the turnover number (Kikuchi et al., 1988). K_{cat} of GTPase activity was determined in the presence or absence of RalGAP (7 μg of protein) as described by Higashijima et al. (1987). K_{ss} for DRal^{S25N} was assayed at 5 μM GTP instead of 1 μM GTP, which was employed for the assay of wild-type DRal and DRal^{G20V}. The K_{cat} of DRal^{S25N} was not determined because most of the γ[^{32}P]GTP was dissociated during these assays. The results shown are the means of three independent experiments.

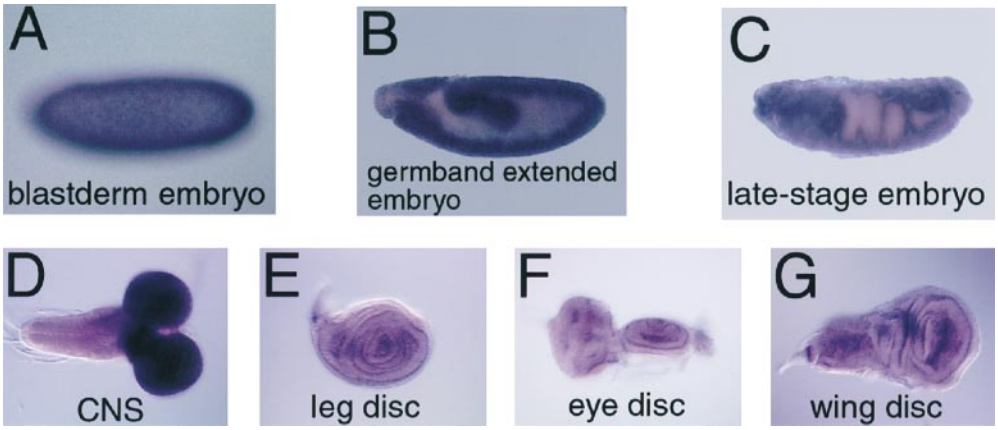

Figure 2. Whole-mount in situ hybridization to wild-type embryos (A–C), central nervous system (CNS; D), and imaginal discs (E–G) with a digoxigenin-labeled *DRal* RNA probe. (A–C) Lateral views of a blastoderm embryo (A), a germband extended embryo (B), and a late-stage embryo (C). The DRal transcripts were detected in almost all the cells in the developing embryos. The mRNA was also detected in the CNS (D), leg disc (E), eye disc (F), and wing disc (G) from third-instar larvae.

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 $DRal^{G20V}$ and $DRal^{S25N}$ resulted in a variety of phenotypes that depended on the GAL4 line used. In this study, we focused on the effect of $DRal^{S25N}$ 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; Adler, 1992; Fristrom et al., 1993; Wong and Adler, 1993). At ~35 h after puparium formation (APF), F-actin accumulates on the distal side of the epithelial cells. Subsequently, outgrowth of prehairs is initiated from the distal side (Wong 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-

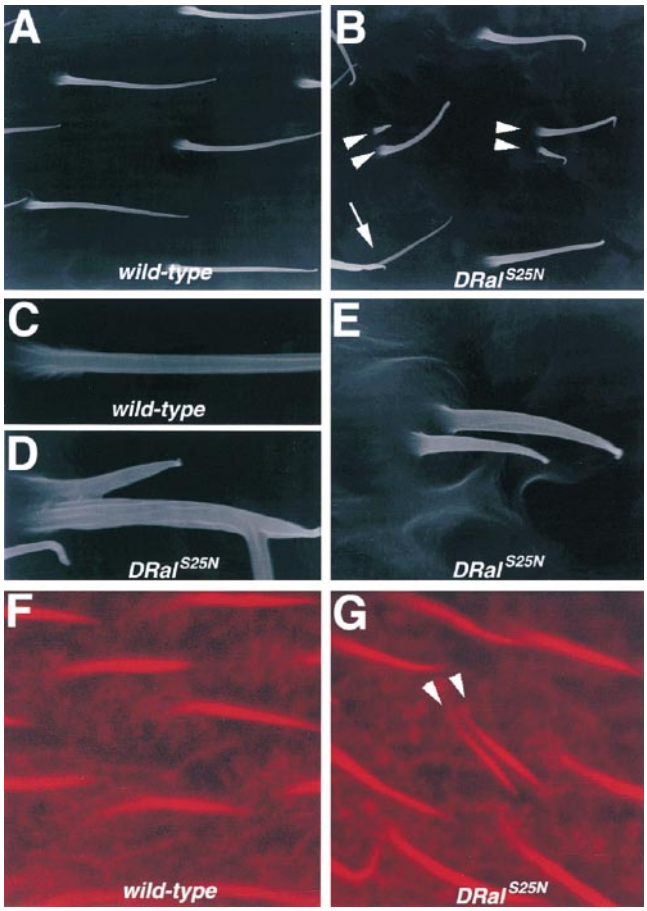

Figure 3. Effects of $DRal^{S25N}$ on the development of wing hairs. (A–E) Surfaces of adult wings were examined with a scanning electron microscope. (A) Wild-type wing. (B) A wing from a fly expressing $DRal^{S25N}$. A forked hair (arrow) and duplicated hairs (arrowheads) are marked. (C) A higher magnification of the hair in A. Higher magnifications of forked (D) and duplicated (E) hairs on $DRal^{S25N}$-expressing wings. (F and G) Prehair development of wild-type and $DRal^{S25N}$-expressing wings from pupae at 30–36 h APF were examined using rhodamine-phalloidin staining. (F) On wild-type wings, single prehairs filled with F-actin elongate from each cell. (G) A pupal wing expressing $DRal^{S25N}$. Two prehairs formed from single cells are marked by arrowheads. Flies analyzed were reared at 29°C.

ity (Fig. 3 A). The hairs on the wings overexpressing the wild-type DRal protein were morphologically indistinguishable from the wild-type hair (data not shown). On the wings expressing DRal^{S25N}, 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 DRal^{S25N}, 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 DRal 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 DRal proteins in the developing trichogens, *UAS-DRal* flies were crossed to the *sca-GAL4* line. The bristles of flies expressing the wild-type DRal 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 DRal^{S25N} resulted in the loss of bristles on the nota (Fig. 4, B and D). In some cases, DRal^{S25N} affected both shafts and sockets, suggesting that DRal may be involved in the development of these structures. Both macrochaetes (large bristles) and microchaetes (small bristles) were affected by the DRal^{S25N} expression.

The absence of bristles on the nota expressing DRal^{S25N} could be due to failure in the process of shaft initiation from the trichogen cells. Alternatively, overexpression of the dominant negative DRal 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 producing a shaft (Fig. 5, A and C). On the nota expressing DRal^{S25N}, the neuron and trichogen were stained with mAb 22C10 in each macrochaete and microchaete, but the developing shafts appeared to be malformed (Fig. 5, B and D). These data indicate that DRal^{S25N} perturbed shaft initiation, but not the recruitment of trichogen cells.

To visualize the F-actin in the developing bristles, pupal nota at 26–32 h APF were dissected and stained with rhodamine-conjugated phalloidin. Fig. 6 A shows developing microchaetes in wild-type nota. Developing shafts containing F-actin were observed at this stage. On the nota expressing DRal^{S25N}, 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 DRal^{S25N}, the development of actin structures in the macrochaetes appeared to be interrupted at the initiation of extension (Fig. 6 D).

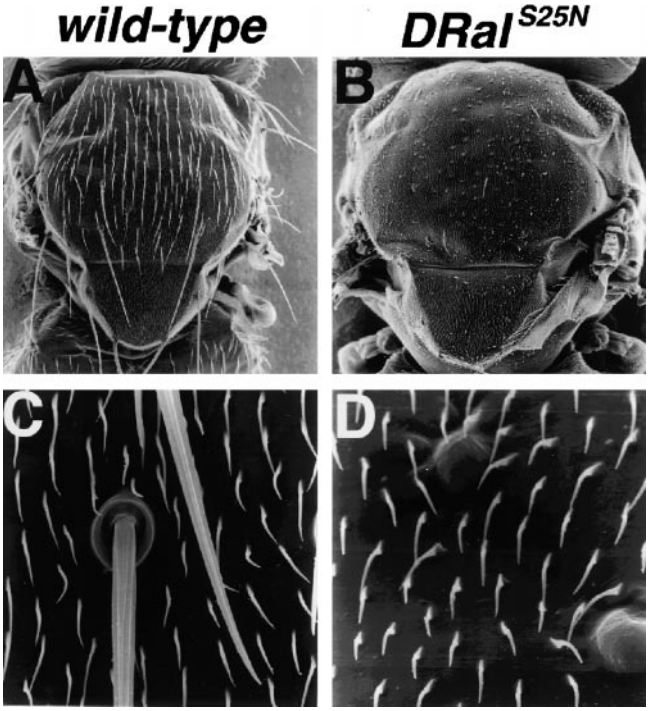

Figure 4. Effects of DRal^{S25N} on bristle development. Adult nota from wild-type (A and C) and DRal^{S25N}-expressing flies (B and D) were examined with a scanning electron microscope. (A and B) Low magnification views. (C and D) High magnification views. (C) The wild-type microchaetes contain one socket and one shaft. (D) Most of the microchaetes expressing DRal^{S25N} contain one socket, but no shaft. Flies analyzed were reared at 29°C.

Next, we used the *GAL4-69B* line to examine the effects of expressing wild-type DRal and DRal^{S25N} 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 DRal^{S25N} 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 DRal protein. To address whether these phenotypes could be caused by decreased function of DRal, wild-type DRal protein was expressed with the dominant negative DRal^{S25N} protein. The loss of bristles and morphological defects resulting from DRal^{S25N} expression were largely rescued by coexpression of the wild-type DRal protein (Fig. 7, B and D). Therefore, these phe-

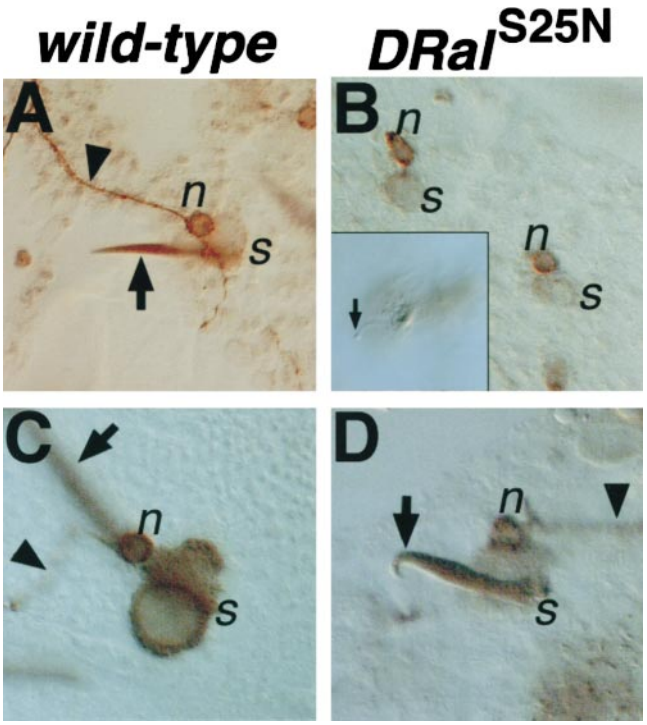

Figure 5. Examination of cell types in microchaetes and macrochaetes by labeling with mAb 22C10. Staining was performed on whole-mount preparations of pupal nota at 26–32 h APF. At the terminal stages of development shown here, the mAb 22C10 (Fujita et al., 1982) stained the cytoplasm of the neuron (n) and axons (arrowheads), and the shaft cell (s) and developing bristles (arrows). (A) Wild-type microchaete. (B) Microchaete from a DRal^{S25N}-expressing cell. The arrow in the inset points to a malformed bristle. (C) Wild-type macrochaete. (D) Macrochaete from a DRal^{S25N}-expressing cell. Flies analyzed were reared at 29°C.

notypes are likely to have resulted from decreased DRal function.

Effects of Mutations in the JNK Pathway on the DRal^{S25N}-induced Phenotype

To explore other genes associated with the DRal-induced defects described above, flies carrying both *sca-GAL4* and *UAS-DRal^{S25N}* 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 DRal^{S25N} (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; Brunner 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 JNKK and JNK dominantly suppressed the DRal^{S25N}-induced bristle phenotype (Fig. 8). Two alleles of the *hemipterous* (*hep*) gene (encoding a JNKK; Glise et al., 1995) and three alleles of the *bas-*

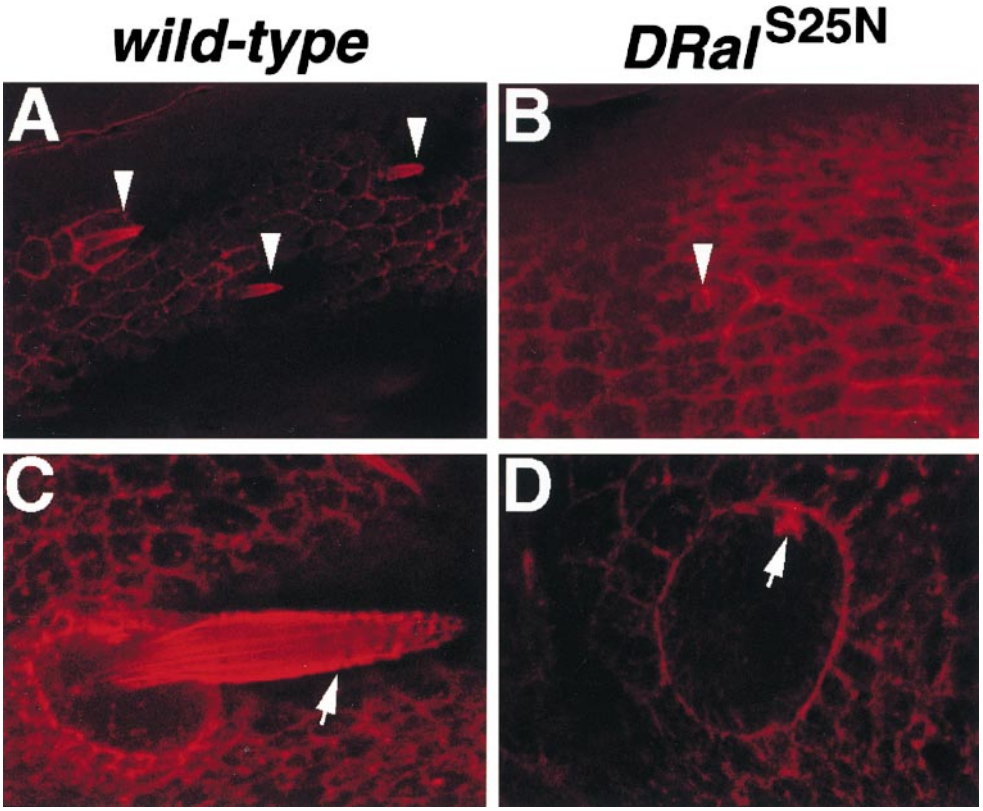

Figure 6. Bristles from 26–32 h APF pupae stained with rhodamine-conjugated phalloidin. (A) Wild-type microchaete. Developing bristles filled with F-actin are marked by arrowheads. (B) Macrochaete from a DRal^{S25N}-expressing cell. A bristle that failed to grow can be identified by the accumulation 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 DRal^{S25N}-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 DRal^{S25N}

Mutant alleles tested	Effects
mbt^{P1}	No effect
mbt^{P2}	No effect
cdc42^{1}	No effect
Df(3L)emc5 (*DRac1^{-}*)	No effect
Df(3L)pbl-X1 (*DRac2^{-}*)	No effect
RhoA^{Df236}	No effect
RhoA^{Df903}	No effect
RhoA^{P2}	No effect
hep^{r75}	Suppression
hep^{1}	Suppression
bsk^{flp147E}	Suppression
bsk^{IIP71}	Suppression
bsk^{1}	Suppression
Ras1^{e2F}	No effect
D-raf^{1}	No effect
Dsor1^{GP158}	No effect
Dsor1^{Su1}	No effect
rl^{EMS64}	No effect
rl^{Su23}	No effect

w^{1118}/Y; *sca-GAL4/sca-GAL4*; *UAS-DRal^{S25N}/MKRS, Sb* males were crossed to females of the indicated mutant stocks. The resulting F1 progenies carrying the mutation indicated, and *sca-GAL4* and *UAS-DRal^{S25N}* were compared with the flies carrying *sca-GAL4* and *UAS-DRal^{S25N}*, but no mutation. Flies analyzed were reared at 29°C.

ket (*bsk*) gene (encoding a JNK; Riesgo-Escovar et al., 1996; Sluss et al., 1996) acted as dominant suppressors of the DRal^{S25N}-induced bristle phenotype (Table II). These genetic interactions suggest that DRal functions in a common signal pathway with JNKK and JNK in an antagonistic fashion.

Effects of DRal^{G20V} on Dorsal Closure

It has been shown that both the *bsk* and *hep* mutations disrupt the process of dorsal closure during embryonic development (Glise 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 *DRal^{G20V}* in the embryonic epidermis, *UAS-DRal^{G20V}* lines were crossed to a number of GAL4 lines such as *actin-GAL4*, *69B-GAL4*, and *arm-GAL4*. Embryonic expression of *DRal^{G20V}* 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, *DRal^{G20V}* was expressed using *pnr-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 DRal^{G20V} 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 (Glise 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-DRal^{G20V}* together with a plasmid that expresses GAL4 under control of the *actin5C* promoter, *pWAGAL4* (Hiromi, Y., unpublished observa-

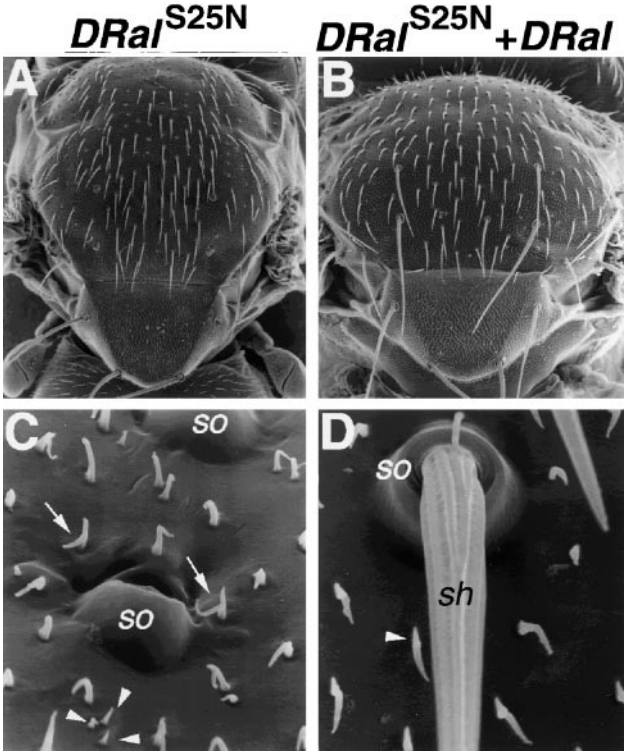

Figure 7. The DRal^{S25N}-induced defects of hairs and bristles are rescued by coexpression with the wild-type DRal protein. Adult nota of flies expressing only DRal^{S25N} (A and C) and flies expressing both DRal^{S25N} and wild-type DRal (B and D) using the *69B* GAL4 driver were examined with a scanning electron microscope. (A and B) Low magnification views. (C and D) High magnification views. (C) Most of the microchaetes expressing DRal^{S25N} contained one socket (so), but no shaft (sh). The hairs were often forked (arrows) and were multiplied (arrowheads). (D) Coexpression of the wild-type DRal protein significantly suppressed the DRal^{S25N}-induced phenotypes. Flies analyzed were reared at 29°C.

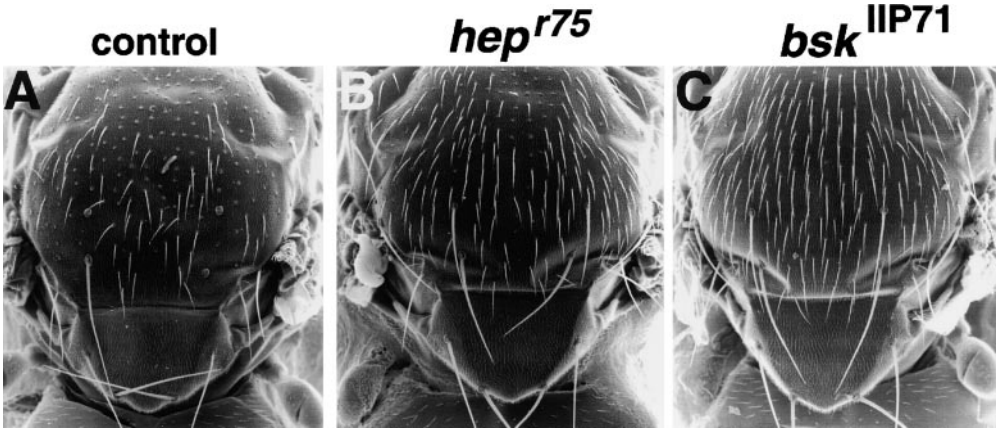

Figure 8. Mutations of the genes encoding JNKK and JNK suppress the DRal^{S25N}-induced bristle phenotype. (A) +/+; *sca-GAL4/CyO*; *UAS-DRal*^{S25N}/+ (control). (B) *hep*^{r75}/+; *sca-GAL4/CyO*; *UAS-DRal*^{S25N}/+ (*hep*^{r75}). (C) +/+; *sca-GAL4/bsk*^{IIP71}; *UAS-DRal*^{S25N}/+ (*bsk*^{IIP71}). The bristle-missing phenotype caused by expression of DRal^{S25N} (A) was dominantly suppressed by the loss of function mutations *hep*^{R75} (B) and *bsk*^{IIP71} (C). For other genotypes, see Table II. Flies analyzed were reared at 29°C.

tions). DRal did not affect the basal level of Bsk phosphorylation in untreated S2 cells (data not shown). It has been shown that JNK is activated by osmotic shock (Galcheva-Gargova et al., 1994). In fact, treatment of the cells with 0.5 M D-sorbitol for 5 min resulted in an increase in Bsk phosphorylation compared with the untreated control (data not shown). Whereas expression of the wild-type DRal protein did not affect Bsk activation, the constitutively active mutant significantly inhibited the phosphorylation of Bsk (Fig. 10). To test whether the difference in the signals determined using the anti-ACTIVE JNK antibody were due to differences in the levels of Bsk protein loaded onto each lane, the blots were probed with an antibody that recognizes total JNK protein (both active and inactive forms), which showed similar signals in each lane (Fig. 10). These results suggest that DRal is an upstream negative regulator of Bsk/JNK in tissue culture cells.

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 sequences. 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; Hariharan 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 Ras inhibits GTP binding and sequesters the GEFs from the endogenous Ras protein (Farnsworth and Feig, 1991). Therefore, the DRal^{S25N} 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 DRal^{S25N}-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-

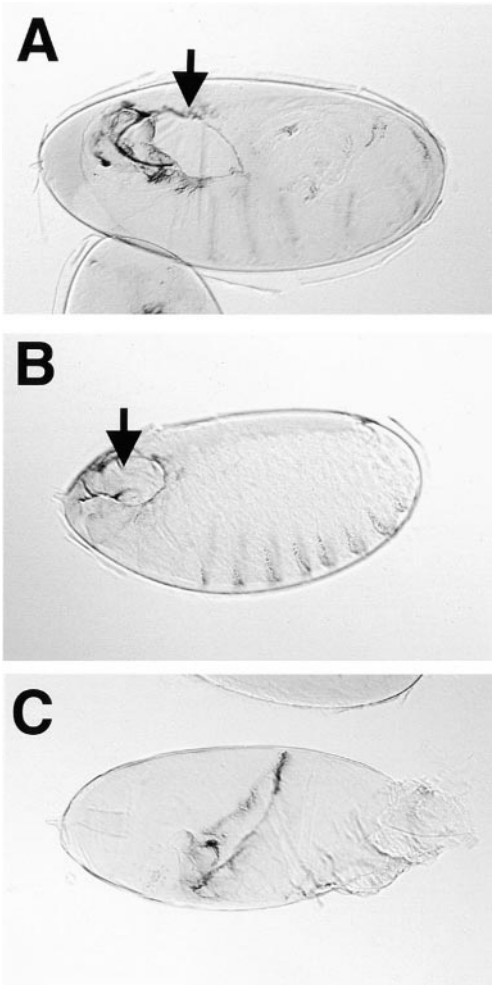

Figure 9. Cuticle phenotype of embryos expressing DRal^{G20V}. (A–C) Dorsolateral views of *pnr-GAL4/UAS-DRal^{G20V}* embryos. Cuticle of the embryos expressing DRal^{G20V} displayed holes (arrows) at the dorsal (A) or anterior (B) surfaces, or a severe dorsal-open phenotype (C). Anterior is to the left.

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 DRal^{S25N} 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 DRal^{S25N} affected their structure. Wing cells that expressed DRal^{S25N} 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 (Cantor et al., 1995; Jullien-Flores et al., 1995; Park and Weinberg, 1995). RalBP1 acts as a GAP for CDC42 and Rac (Cantor 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 DRal^{S25N}-induced phenotype could be suppressed by halving the dosages of Hep/JNKK and Bsk/JNK. Ex-

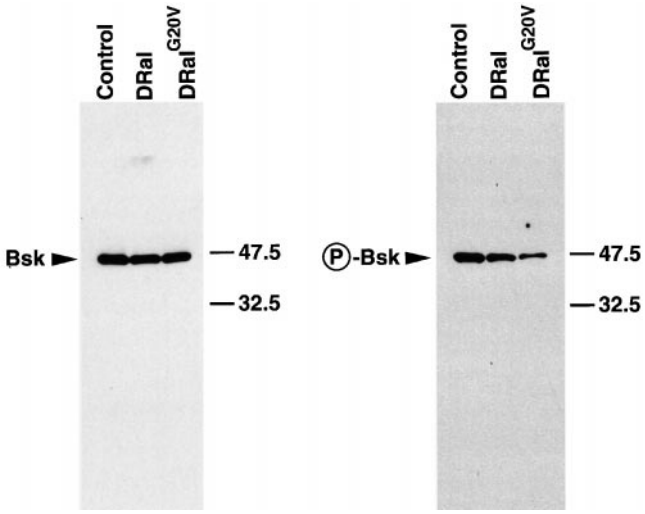

Figure 10. Expression of DRal^{G20V} inhibits sorbitol-induced JNK phosphorylation. Western blot analysis of cell extracts from S2 cells that were transfected with *pWAGAL4* alone (Control), *pWAGAL4* plus *pUAST-DRal* (DRal), or *pWAGAL4* plus *pUAST-DRal^{G20V}* (DRal^{G20V}). Left, the Bsk protein was detected using an anti-JNK1 antibody. Expression of DRal and DRal^{G20V} did not affect the amount of the Bsk protein. Right, phosphorylation of Bsk was detected using an antibody that specifically recognizes phosphorylated JNK. DRal^{G20V} 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 $DRal^{G20V}$ 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 *DCdc42*, *DRac1*, *DRac2*, and *RhoA* on the $DRal^{S25N}$-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 Ral (Vojtek and Der, 1998). Ras mediates its effects on cellular proliferation in part by activating Raf (reviewed by Bos, 1997; Vojtek and Der, 1998). Ras is also known to have effects on the cytoskeleton (Bar Sagi and Feramisco, 1986; Ridley and Hall, 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 Ral, which is activated by another family of Ras effectors, the RalGEFs, also regulates the cytoskeleton through the JNK pathway, and thus plays a role in the cell shape changes that occur in animal development.

We would like to thank Norbert Perrimon, Kozo Kaibuchi, and Beth Stronach for comments on the manuscript and valuable discussions. We are grateful to Richard G. Fehon, Mathew Freeman, Toshihiko Hosoya, Fumio Matsuzaki, Marek Mlodzik, Makoto Nakamura, Yasuyoshi Nishida, Masataka Okabe, Norbert Perrimon, Thomas Raabe, Gerald M. Rubin, Kuniaki Takahashi, Yoshihiro Takatsu, Minoru Tateno, and Ryu Ueda. The Bloomington Stock Center for fly stocks, Shinobu C. Fujita for the mAb 22C10, Yasushi Hiromi for the *pWAGAL4* plasmid, Hiroko Kouike for sequencing, Ritsuko Shimamura for making the fly medium, and Ken-ichi Kimura, Ryusuke Niwa, Masataka Okabe, Kuniaki Takahashi, Minoru Tateno, and Tadashi Uemura for technical instructions.

This work was supported by grants from the Japanese Ministry of Science, Education, Sports, and Culture to K. Sawamoto, A. Kikuchi, and H. Okano. H. Okano was also supported by the Human Frontier Science Program Organization and Core Research for Evolutional Science and Technology, Japan Science and Technology Corporation.

Submitted: 25 January 1999
Revised: 19 May 1999
Accepted: 10 June 1999

References

Adler, P.N. 1992. The genetic control of tissue polarity in *Drosophila*. *BioEssays*. 14:735–741.

Albright, C.F., B.W. Giddings, J. Liu, M. Vito, and R.A. Weinberg. 1993. Characterization of a guanine nucleotide dissociation stimulator for a ras-related GTPase. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:339–347.

Appel, L.F., M. Prout, R. Abu-Shumays, A. Hammonds, J.C. Garbe, D. Fristrom, and J. Fristrom. 1993. The *Drosophila stubble-stubbloid* gene encodes an apparent transmembrane serine protease required for epithelial morphologenesis. *Proc. Natl. Acad. Sci. USA*. 90:4937–4941.

Bar Sagi, D., and J.R. Feramisco. 1986. Induction of membrane ruffling and fluid-phase pinocytosis in quiescent fibroblasts by ras proteins. *Science.* 233: 1061–1068.

Barbacid, M. 1987. ras genes. *Annu. Rev. Biochem*. 56:779–827.

Barrett, K., M. Leptin, and J. Settleman. 1997. The Rho GTPase and a putative RhoGEF mediate a signaling pathway for the cell shape changes in *Drosophila* gastrulation. *Cell*. 91:905–915.

Bos, J.L. 1997. Ras-like GTPases. *Biochem. Biophys. Acta* 1333:M19–M31.

Brand, A.H., and N. Perrimon. 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development.* 118: 401–415.

Brunner, D., N. Oellers, J. Szabad, W.H. Biggs III, S.L. Zipursky, and E. Hafen. 1994. A gain-of-function mutation in *Drosophila* MAP kinase activates multiple receptor tyrosine kinase signaling pathways. *Cell.* 76:875–888.

Cant, K., B.A. Knowles, M.S. Mooseker, and L. Cooley. 1994. *Drosophila singed*, a facin homolog, is required for actin bundle formation during oogenesis and bristle extension. *J. Cell Biol*. 125:369–380.

Cantor, S.B., T. Urano, and L.A. Feig. 1995. Identification and characterization of Ral-binding protein 1, a potential downstream target of Ral GTPase. *Mol. Cell. Biol*. 15:4578–4584.

Chardin, P., and A. Tavitian. 1989. Coding sequences of human ralA and ralB cDNAs. *Nucleic Acid Res.* 17:4380.

Coso, O.A., M. Chiariello, J.C. Yu, H. Teramoto, P. Crespo, N. Xu, T. Miki, and J.S. Gutkind. 1995. The small GTP-binding proteins Rac and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. *Cell.* 81:1137–1146.

Eaton, S., R. Wept, and K. Simons. 1996. Roles of Rac1 and Cdc42 in planar polarization and hair outgrowth in the wing of *Drosophila*. *J. Cell Biol*. 135: 1277–1289.

Emkey, R., S. Freedman, and L.A. Feig. 1991. Characterization of a GTPase-activating protein for the Ras-related Ral protein. *J. Biol. Chem*. 266:9703–9706.

Farnsworth, C.L., and L.A. Feig. 1991. Dominant inhibitory mutations in the Mg^{2+} binding site of Ras blocks its activation by GTP. *Mol. Cell. Biol*. 11: 4822–4829.

Fehon, R.G., T. Oren, D.R. LaJeunesse, T.E. Melby, and B.M. McCartney. 1997. Isolation of mutations in the *Drosophila* homologues of the human Neurofibromatosis 2 and yeast CDC42 genes using a simple and efficient reverse-genetic method. *Genetics.* 146:245–252.

Feig, L.A., and G.M. Cooper. 1988. Inhibition of NIH 3T3 cell proliferation by a mutant ras protein with preferential affinity for GDP. *Mol. Cell. Biol*. 8:3235–3243.

Feig, L.A., T. Urano, and S. Cantor. 1996. Evidence for a Ras/Ral signaling cascade. *Trends Biochem. Sci.* 21:438–441.

Fisher-Vize, J.A, G.M. Rubin, and R. Lehmann. 1992. The *fat facets* gene is required for *Drosophila* eye and embryo development. *Development.* 116:985–1000.

Fristrom, D., M. Wilcox, and J. Fristrom. 1993. The distribution of PS integrins, laminin A and F-actin during key stages in *Drosophila* wing development. *Development*. 117:509–523.

Fujita, S.C., S.L. Zipursky, S. Benzer, A. Ferrus, and S.L. Shotwell. 1982. Monoclonal antibodies against the *Drosophila* nervous system. *Proc. Natl. Acad. Sci. USA*. 79:7929–7933.

Galcheva-Gargova, Z., B. Derijard, I.H. Wu, and R.J. Davis. 1994. An osmosensing signal transduction pathway in mammalian cells. *Science*. 265:806–808.

Glise, B., and S. Noselli. 1997. Coupling of Jun amino-terminal kinase and decapentaplegic signaling pathways in *Drosophila* morphogenesis. *Genes Dev*. 11:1738–1747.

Glise, B., H. Bourbon, and S. Noselli. 1995. *hemipterous* encodes a novel *Drosophila* MAP kinase kinase, required for epithelial cell sheet movement. *Cell*. 83:451–461.

Goi, T., G. Rusanescu, T. Urano, and L.A. Feig. 1999. Ral-specific guanine nucleotide exchange factor activity opposes other Ras effectors in PC12 cells by inhibiting neurite outgrowth. *Mol. Cell. Biol*. 19:1731–1741.

Hacker, U., and N. Perrimon. 1998. *DRhoGEF2* encodes a member of the Dbl family of oncogenes and controls cell shape changes during gastrulation in *Drosophila*. *Genes Dev*. 12:274–284.

Harden, N., H.Y. Loh, W. Chia, and L. Lim. 1995. A dominant inhibitory version of the small GTP-binding protein Rac disrupts cytoskeletal structures and inhibits developmental cell shape changes in *Drosophila*. *Development.* 121:903–914.

Harden, N., J. Lee, H.-Y. Loh, Y.M. Ong, I. Tan, T. Leung, E. Manser, and L. Lim. 1996. A *Drosophila* homolog of the Rac- and Cdc42-activated serine/threonine kinase PAK is a potential focal adhesion and focal complex protein that colocalizes with dynamic actin structures. *Mol. Cell. Biol.* 16:1896–1908.

Hariharan, I.K., R.W. Carthew, and G.M. Rubin. 1991. The *Drosophila roughened* mutation: activation of a rap homolog disrupts eye development and interferes with cell determination. *Cell*. 67:717–722.

Hariharan, I.K., K.-Q. Hu, H. Asha, A. Quintanilla, R.M. Ezzell, and J. Settleman. 1995. Characterization of rho GTPase family homologues in *Drosophila melanogaster*: overexpressing *Rho1* in retinal cells causes a late developmental defect. *EMBO (Eur. Mol. Biol. Organ.) J.* 14:292–302.

Hartenstein, V., and J.W. Posakony. 1989. Development of adult sensilla on the wing and notum of *Drosophila melanogaster*. *Development.* 107:389–405.

Higashijima, T., K.M. Ferguson, P.C. Sternweis, M.D. Smigel, and A.G. Gilman. 1987. Effects of Mg^{2+} and the beta gamma-subunit complex on the interactions of guanine nucleotides with G proteins. *J. Biol. Chem*. 262:757–761.

Hinoi, T., S. Kishida, S. Koyama, M. Ikeda, Y. Matsuura, and A. Kikuchi. 1996. Post-translational modifications of Ras and Ral are important for the action of Ral GDP dissociation stimulator. *J. Biol. Chem*. 271:19710–19716.

Hofer, F., S. Fields, C. Schneider, and G.S. Martin. 1994. Activated Ras interacts with the Ral guanine nucleotide dissociation stimulator. *Proc. Natl. Acad. Sci. USA*. 91:11089–11093.

Hopmann, R., J.A. Cooper, and K.G. Miller. 1996. Actin organization, bristle morphology, and viability are affected by actin capping protein mutations in *Drosophila*. *J. Cell Biol.* 133:1293–1305.

Jiang, H., J.-Q. Luo, T. Urano, P. Frankel, Z. Lu, D.A. Foster, and L.A. Feig. 1995. Involvement of Ral GTPase in v-Src-induced phospholipase D activation. *Nature*. 378:409–412.

Jullien-Flores, V., O. Dorseuil, F. Romero, F. Letourneur, S. Saragosti, R. Berger, A. Tavitian, G. Gacon, and H.J. Camonis. 1995. Bridging Ral GTPase to Rho pathways. RLIP76, a Ral defector with CDC42/Rac GTPase-activating protein activity. *J. Biol. Chem*. 270:22473–22477.

Kahn, S., F. Yamamoto, C. Almoguera, E. Winter, K. Forrester, J. Jordano, and M. Perucho. 1987. The c-K-ras gene and human cancer. *Anticancer Res.* 7:639–652.

Kikuchi, A., T. Yamashita, M. Kawata, K. Yamamoto, K. Ikeda, T. Tanimoto, and Y. Takai. 1988. Purification and characterization of a novel GTP-binding protein with a molecular weight of 24,000 from bovine brain membranes. *J. Biol. Chem*. 263:2897–2904.

Kikuchi, A., S.D. Demo, Z.-H. Ye, Y.W. Chen, and L.T. Williams. 1994. ral-GDS family members interact with the effector domain of ras p21. *Mol. Cell. Biol.* 14:7483–7491.

Kishida, S., S. Koyama, K. Matsubara, M. Kishida, Y. Matsuura, and A. Kikuchi. 1997. Colocalization of Ras and Ral on the membrane is required for Ras-dependent Ral activation through Ral GDP dissociation stimulator. *Oncogene.* 15:2899–2907.

Luo, L, Y.J. Liao, L.Y. Jan, and Y.N. Jan. 1994. Distinct morphogenetic functions of similar small GTPases: *Drosophila* DRac1 is involved in axonal outgrowth and myoblast fusion. *Genes Dev*. 8:1787–1802.

Melzig, J., K.-H. Rein, U. Schafer, H. Pfister, H. Jackle, M. Heisenberg, and T. Raabe. 1998. A protein related to p21-activated kinase (PAK) that is involved in neurogenesis in the *Drosophila* adult central nervous system. *Curr. Biol.* 8:1223–1226.

Miller, M.J., S. Prigent, E. Kupperman, L. Rioux, S.H. Park, J.R. Feramisco, M.A. White, J.L. Rutkowski, and J.L. Meinkoth. 1997. RalGDS functions in Ras- and cAMP-mediated growth stimulation. *J. Biol. Chem*. 272:5600–5605.

Mitchell, H.K., J. Roach, and N.S. Petersen. 1983. The morphogenesis of cell hairs on Drosophila wings. *Dev. Biol.* 95:387–398.

Moscow, J.A., R. He, J.M. Gudas, and K.H. Cowan. 1994. Utilization of multiple polyadenylation signals in the human RHOA protooncogene. *Gene.* 144: 229–236.

Murai, H., M. Ikeda, S. Kishida, O. Ishda, M. Okazaki-Kishida, Y. Matsuura, and A. Kikuchi. 1997. Characterization of Ral GDP dissociation stimulator-like (RGL) activities to regulate c-fos promoter and the GDP/GTP exchange of Ral. *J. Biol. Chem*. 272:10483–10490.

Neuman-Silberberg, F.S., E. Schejter, F.M. Hoffmann, and B.Z. Shilo. 1984. The Drosophila ras oncogenes: structure and nucleotide sequence. *Cell.* 37: 1027–1033.

Nishida, Y., M. Hata, T. Ayaki, H. Ryo, M. Yamagata, K. Shimizu, and Y. Nishizuka. 1988. Proliferation of both somatic and germ cells is affected in the *Drosophila* mutants of *raf* proto-oncogene. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:775–781.

Noselli, S. 1998. JNK signaling and morphogenesis in *Drosophila*. *Trends Genet.* 14:33–38.

Okazaki, M., S. Kishida, T. Hinoi, T. Hasegawa, M. Tamada, T. Kataoka, and A. Kikuchi. 1997. Synergistic activation of c-fos promoter activity by Raf and RalGDP dissociation stimulator. *Oncogene.* 14:515–521.

Overton, J. 1967. The fine structure of developing bristles in wild-type and mutant *Drosophila melanogaster*. *J. Morphol.* 122:367–380.

Park, S.H., and R.A. Weinberg. 1995. A putative effector of Ral has homology to Rho/Rac GTPase activating proteins. *Oncogene.* 11:2349–2355.

Petersen, N.S., D.-H. Lankenau, H.K. Mitchell, P. Young, and V.G. Corces. 1994. Forked proteins are components of fiber bundles present in developing bristles of *Drosophila melanogaster*. *Genetics.* 136:173–182.

Ridley, A.J., and A. Hall. 1992. The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell.* 70:389–399.

Riesgo-Escovar, J.R., M. Jenni, A. Fritz, and E. Hafen. 1996. The *Drosophila* Jun-N-terminal kinase is required for cell morphogenesis but not for Djun-dependent cell fate specification in the eye. *Genes Dev*. 10:2759–2768.

Rodriguez-Viciana, P., P.H. Warne, A. Khwaja, B.M. Marte, D. Pappin, P. Das, M.D. Waterfield, A. Ridley, and J. Downward. 1997. Role of phosphoinositide 3-OH kinase in cell transformation and control of the actin cytoskeleton by Ras. *Cell.* 89:457–467.

Sawamoto, K., H. Okano, Y. Kobayakawa, S. Hayashi, K. Mikoshiba, and T. Tanimura. 1994. The function of argos in regulating cell fate decisions during the *Drosophila* eye and wing vein development. *Dev. Biol.* 164:267–276.

Schweighoffer, F., H. Cai, M.C. Chevallier-Multon, I. Fath, G. Cooper, and B. Tocque. 1993. The *Saccharomyces cerevisiae* SDC25 C-domain gene product overcomes the dominant inhibitory activity of Ha-Ras asn-17. *Mol. Cell. Biol.* 13:39–43.

Shoji, I., A. Kikuchi, S. Kuroda, and Y. Takai. 1989. Kinetic analysis of the binding of guanine nucleotide to bovine brain smg p25A. *Biochem. Biophys. Res. Commun*. 162:273–281.

Simon, M.A., D.D. Bowtell, G.S. Dodson, T.R. Laverty, and G.M. Rubin. 1991. Ras1 and a putative guanine nucleotide exchange factor perform crucial steps in signal transduction by the sevenless protein tyrosine kinase receptor. *Cell.* 67:701–716.

Sluss, H.K., Z. Han, T. Barrett, R.J. Davis, and Y.T. Ip. 1996. A JNK signal transduction pathway that mediates morphogenesis and an immune response in *Drosophila*. *Genes Dev*. 10:2745–2758.

Spaargaren, M., and J.R. Bischoff. 1994. Identification of the guanine nucleotide dissociation stimulator for Ral as a putative effector molecule of R-ras, H-ras, K-ras and rap. *Proc. Natl. Acad. Sci. USA* 91:12609–12613.

Strutt, D.I., U. Weber, and M. Mlodzik. 1997. The role of RhoA in tissue polarity and Frezzled signalling. *Nature*. 387:292–295.

Tautz, D., and C. Pfeifle. 1989. A non-radioactive in situ hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene *hunchback*. *Chromosoma.* 98:81–85.

Tilney, L.G., M.S. Tilney, and G.M. Guild. 1995. Actin bundles in *Drosophila* bristles. I. Two filament cross-linked are involved in bundling. *J. Cell Biol*. 130:629–638.

Tilney, L.G., P. Connelly, S. Smith, and G.M. Guild. 1996. F-actin bundles in *Drosophila* bristles are assembled from modules composed of short filaments. *J. Cell Biol.* 135:1291–1308.

Tsuda, L., Y.H. Inoue, M. Yoo, M. Mizuno, M. Hata, Y. Lim, T. Adachi-Yamada, Y. Ryo, Y. Masamune, and Y. Nishida. 1993. A protein kinase similar to MAP kinase activator acts downstream of the raf kinase in *Drosophila*. *Cell.* 72:407–414.

Turner, C.M., and P.N. Adler. 1998. Distinct roles for the actin and microtubule cytoskeletons in the morphogenesis of epidermal hairs during wing development in *Drosophila*. *Mech. Dev*. 70:181–192.

Urano, T., R. Emkey, and L.A. Feig. 1996. Ral-GTPases mediates a distinct downstream signaling pathway from Ras that facilitates cellular transformation. *EMBO (Eur. Mol. Biol. Organ.) J.* 15:810–816.

Van Aelst, L., and C. D'Souza-Schorey. 1997. Rho GTPases and signaling networks. *Genes Dev*. 11:2295–2322.

Verheyen, E.M., and L. Cooley. 1994. Profilin mutations disrupt multiple actin-dependent processes during *Drosophila* development. *Development.* 120: 717–728.

Vojtek, A.B., and C.J. Der. 1998. Increasing complexity of the Ras signaling pathway. *J. Biol. Chem*. 273:19925–19928.

Wes, P.D., M. Yu, and C. Montell. 1996. RIC, a calmodulin-binding Ras-like GTPase. *EMBO (Eur. Mol. Biol. Organ.) J.* 15:5839–5848.

White, M.A., T. Vale, J.H. Camonis, E. Schaefer, and M.H. Wigler. 1996. A role for the Ral guanine nucleotide dissociation stimulator in mediating Ras-induced transformation. *J. Biol. Chem*. 271:16439–16442.

Wieschaus, E., and C. Nüsslein-Volhard. 1986. Looking at embryos. *In Drosophila,* A Practical Approach. D.B. Roberts, editor. IRL Press, Oxford/ Washington, D.C. 199–227.

Wolthuis, R.M., B. Bauer, L.J. van't Veer, A.M. de Vries-Smits, R.H. Cool, M. Spaargaren, A. Wittinghofer, B.M. Burgering, and J.L. Bos. 1996. RalGDS-like factor (Rlf) is a novel Ras and Rap 1A-associating protein. *Oncogene.* 13:353–362.

Wolthuis, R.M., N.D. de Ruiter, R.H. Cool, and J.L. Bos. 1997. Stimulation of gene induction and cell growth by the Ras effector Rlf. *EMBO (Eur. Mol. Biol. Organ.) J.* 22:6748–6761.

Wolthuis, R.M., F. Zwartkruis, T.C. Moen, and J.L. Bos. 1998. Ras-dependent activation of the small GTPase Ral. *Curr. Biol.* 8:471–474.

Wong, L.L., and P.N. Adler. 1993. Tissue polarity genes of *Drosophila* regulate the subcellular location for prehair initiation in pupal wing cells. *J. Cell Biol.* 123:209–221.

Wulfkuhle, J.D., N.S. Petersen, and J.J. Otto. 1998. Changes in the F-actin cytoskeleton during neurosensory bristle development in Drosophila: the roles of singed and forked proteins. *Cell Motil. Cytoskelet.* 40:119–132.

Zuker, C.S., A.F. Cowman, and G.M. Rubin. 1985. Isolation and structure of a rhodopsin gene from *D. melanogaster*. *Cell.* 40:851–858.