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Molecular and Functional Characterization of a Unique Rab Protein, RABRP1, Containing the WDIAGQE Sequence in a GTPase Motif

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ABSTRACT—Rab proteins of the small G-protein superfamily are known to be involved in intracellular vesicle transport. Here, we describe the unique characteristics of a novel Rab protein, RABRP1 (Rab-Related Protein 1). The *Drosophila* RabRP1 gene is mainly transcribed in the eyes and testes, where the 3-kb and 1.5-kb mRNAs, respectively, are the predominant gene products. The amino-acid sequence deduced from the longer cDNA indicated that the C-terminal 1/3 of the sequence shares homology with Rab proteins, whereas the rest of the peptide shows no significant homology with any other proteins. Immunoblot analysis using antiserum against the Rab-domain indicated that the multiple translates (94 k, 53 k, 30 k, 29 k and 27 k) were expressed in the eyes. In contrast, only smaller peptides (30 k, 29 k and 27 k) were identified in the testes. Molecular phylogenetic analysis revealed that RABRP1 forms a subgroup with *Dictiostelium* RabE and mammalian Rab29, Rab32, Rab38 proteins, whose functions have not been identified yet. RABRP1 and its relatives were characterized by the amino acid substitution occurring in the conserved GTP-binding motifs. Immunohistochemical studies demonstrated that RABRP1 was localized on the subrhabdomeric cisternae of photoreceptor cells and on the pigment granules in photoreceptor and pigment cells in the retina. The expression of the dominant negative RABRP1 caused the abnormal accumulation of autophagosome-like vesicles. These data suggest that RABRP1 is involved in the lysosomal vesicle transport pathway, including the biogenesis or degradation of pigment granules.

Key words: Rab, photoreceptor cell, pigment cell, pigment granule, *Drosophila melanogaster*

INTRODUCTION

Proteins newly synthesized in the rER are transported to their functioning sites through several intracellular compartments (exocytosis). Likewise, extracellular nutrients and prostrate proteins on the plasma membrane are incorporated into the intracellular organelles (endocytosis). In both, transport of the molecules is usually mediated by vesicles, which bud from donor membranes and then recognize and fuse precisely with the target membranes. Rab proteins, members of a small GTP-binding protein superfamily, play a crucial role in regulation of this vesicle transport pathway (Novick and Brennwald, 1993). In mammals, more than 60 Rab proteins have been identified (Pereira-Leal and Seabra, 2000, 2001). Each Rab protein is located in a distinct subcellular compartment and is believed to regulate a particular stage of vesicle transport (Novick and Zerial, 1997; Simons and Zerial, 1993). For example, RAB1 and RAB8 are engaged in transport from the rER to the *cis*-Golgi (Tisdale *et al*., 1992) and from the *trans*-Golgi to the basolateral plasma membrane (Huber *et al*., 1993), respectively. On the other hand, RAB5 is involved in the endocytic pathway from the coated pit of the plasma membrane to the early endosome (Bucci *et al*., 1992).

Molecular cloning and sequencing of various Rab proteins from yeast and mammalian cells revealed that 5 specific motifs of amino-acid sequences are conserved in most Rab proteins. Four of them are essential for the proteins to bind and hydrolyze GTP, and are also conserved in other subfamilies of small GTP-binding proteins. The other motif, often referred to as the “effector region”, is conserved within the Rab protein family, and is implicated in binding to a target protein. Since the pioneering study of Satoh et al. (Satoh *et al*., 1997b), 14 cDNA clones of Rab family proteins have been identified from *Drosophila melanogaster*. Among them,

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10 kinds of Rab proteins share high degrees (> 80%) of sequence homology with the corresponding mammalian Rab proteins, and would most likely be *Drosophila* homologs of those Rab proteins. In contrast, the other 4 Rab proteins, which are referred to as Rab-related proteins (RABRPs), showed low degrees of homology with the already-known Rab proteins, although they also contained the conserved motifs mentioned above (Satoh *et al.*, 1997b). One of them, RABRP1, has particularly unique features not observed in other Rab proteins. A recent *Drosophila* genome project suggested that the transcript of RabRP1 gene possibly has an unusually long open reading frame encoding 686 amino acids (Adams *et al.*, 2000). The molecular weight of RABRP1 estimated from the sequence is 74.7k, which is much higher than those of other Rab proteins (20–30 k). However, the size of the protein actually expressed and functioning *in vivo* remains unclear. Also, RABRP1 carries an amino acid substitution of isoleucine for threonine that locates in the second motif for GTP binding and hydrolysis (WD**T**AGQE → WD**I**AGQE). This threonine residue is highly conserved in most small G-proteins, but a few members of Rab protein (*Dictiostelium* RabE, mammalian Rab29, Rab32 and Rab38), whose functions *in vivo* have not been elucidated, also show this unique substitution (Jager *et al.*, 2000; Norian *et al.*, 1999). Because these Rab proteins including *Drosophila* RABRP1 are clustered in a molecular phylogenetic tree, detailed characterization of RABRP1 will certainly be helpful to elucidate the common features and functions among these unique Rab proteins.

If a novel Rab protein showed some tissue-specific distribution, it could provide valuable information for elucidating the role of the protein. For example, RAB3A is distributed in neurons, and specifically functions in the docking and fusion of synaptic vesicles (Fischer von Mollard *et al.*, 1990; Geppert *et al.*, 1994). RAB17 exists exclusively in the epithelial cells, and is suggested to function in the formation of the cell polarity (Lutcke *et al.*, 1993; Zacchi *et al.*, 1998). Furthermore, the analysis of the morphological and physiological effects induced by the inhibition of a Rab protein activity *in vivo* would provide precise knowledge of the function of the protein (Satoh *et al.*, 1997a). In the present study, we therefore examined the distribution of RABRP1 using immunochemical and immunohistochemical techniques. In addition, we investigated the effect of the expression of a dominant negative RabRP1 protein that can interfere with the native RABRP1 function.

MATERIALS AND METHODS

Fly stocks

All experiments were carried out on white-eyed (w) *Drosophila melanogaster* (*Oregon R, A35 or w1118*). For immunohistochemistry, we also used wild-type flies (*Canton S*). Flies were raised in a room kept at 25°C with a 12 hr light / 12 hr dark cycle of fluorescent lighting at an intensity of 50 lux.

Cloning and sequencing of RabRP1 cDNA

Cloning and sequencing of RabRP1 cDNA were performed according to a method described previously (Satoh *et al.*, 1997b). Briefly, cDNA fragments partially encoding RabRP1 were amplified with polymerase chain reaction (PCR) from a pool of single-stranded DNA reverse-transcribed from *Drosophila* retinal mRNA. Two oligonucleotide primers for amplification were designed at the effector region and the GTP-binding region III of Rab protein. The amplified cDNA fragments of ca. 240 bp were subcloned in pUC18 vector DNA, and sequenced by the dideoxy chain termination method using a Taq Dye Primer Cycle Sequencing Kit and a 373A DNA sequencer (Applied Biosystems, USA). Using the cloned RabRP1 cDNA fragment as a probe, we screened a *Drosophila* head cDNA library (Satoh *et al.*, 1997b). We obtained several positive clones in pBluescript II SK+ vector DNA (Stratagene, USA), and sequenced as described above. Although the longest cDNA encoded the whole region of the Rab-domain of RabRP1, it is still insufficient to cover the complete coding region of RabRP1 expected from the genomic sequence provided by the *Drosophila* genome project. Therefore, we carried out the 5'RACE method using a single stranded cDNA pool as a template in order to obtain the 5'-end sequence of RabRP1.

Northern hybridization

Approximately 900 flies (0–7 days after eclosion) were frozen in liquid nitrogen and shaken vigorously in a plastic tube to separated heads from bodies. After dehydration in cold acetone (–30°C) for 10 days, retinas, brains, ovaries and testes were dissected out under a dissection microscope. Poly(A)$^+$ RNA was extracted separately from each tissue or organ using a QuickPrep Micro mRNA Purification Kit (Amersham Pharmacia Biotech, UK), and roughly quantified spectrophotometrically. Poly(A)$^+$ RNA was then separated on a 1.4% agarose gel containing 6% formaldehyde, vacuum transferred onto a nylon membrane (Hybond-N, Amersham Pharmacia Biotech, UK) and fixed on the membrane by u.v. irradiation. We used a cDNA fragment of RabRP1 (ca. 1 kb) as a probe. The fragment consists of cDNA encoding the Rab-domain of RabRP1 and a part of the 3'-noncoding region. Hybridization was carried out at 53°C for 12hr, and the membrane was washed with 2×SSC at room temperature, followed by washing with 0.2×SSC containing 0.1% SDS at 60°C. Signals were detected with an X-ray film (X-Omat AR, Kodak, USA). After removing the signals by severe washing, we reused the filter for another hybridization with a control probe (histone cDNA probe).

Preparation of anti-RABRP1 antiserum

The cDNA fragment encoding the Rab-domain of RABRP1 was recloned into the pQE60 expression vector (Qiagen, Germany). The fusion protein carrying a 6×histidine tag at the C-terminal of RABRP1 was expressed in *E. coli* (JM109) cells. N-terminal 17 amino-acid residues of Rab-domain were eliminated to increase the yield of the fusion protein. The fusion protein was purified with Ni-NTA agarose resin (Qiagen, Germany) and injected into mice. Antisera against RABRP1 were raised and collected as has been described (Satoh *et al.*, 1997a).

SDS-PAGE and immunoblotting

Flies (0–7 days after eclosion) were rapidly frozen in liquid nitrogen and dehydrated in cold acetone (–30°C) for 10 days. Dehydrated eyes, brains, thoraxes, and testis were isolated as described above. Each tissue was dissolved with SDS-containing buffer, and solubilized proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (Laemmli, 1970). The concentration of acrylamide was 5% in the stacking gel and 12.5% in the separating gel. After electrophoresis, proteins were electrophoretically blotted onto polyvinylidene difluoride (PVDF) membrane (Millipore, USA), and incubated with anti-

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RABRP1 antiserum at 4°C for overnight. Immunoreactive proteins were detected using an avidin-biotin amplification system (Vector Laboratories, USA).

Immunofluorescence microscopy

Compound eyes were dissected out of flies with a razor blade. The eyes were immediately fixed with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS) at pH7.4 (on ice, 2 hr), and washed with a graded series of sucrose-containing phosphate buffers (SPB) (5, 10, 15, 20% (W/V) sucrose in 0.1 M PBS), each step taking 4–12 hr at 4°C. The fixed eyes were embedded in a mixture of Tissue-Tek OCT Compound (Sakura, Japan) and 20% SPB (1:1), and frozen in liquid nitrogen mediated with isopentane. Cryosections 15-mm thick were mounted on albumin-coated glass slides (Matsunami, Japan), and air-dried. The sections were incubated overnight at 4°C with anti-RABRP1 antiserum diluted 1:100 in TTBS (0.1M Tris-Cl, 0.9% NaCl, 0.1% Tween20, pH7.5), and rinsed with TTBS at 20°C (10 min × 6). Sections were then incubated with biotinylated horse anti-mouse IgG for 4 hours at 4°C, and rinsed as above. The specimens were further incubated with FITC-conjugated streptavidin for 1 hr at room temperature, and green fluorescence of FITC was observed with a confocal scanning microscope (MRC-1024; Bio-Rad Laboratories, USA).

Conventional electron and immunoelectron microscopy

Conventional electronmicroscopy was carried out using a method described previously (Satoh *et al*., 1997a). In brief, compound eyes were dissected out of the flies (3-days after eclosion), and immediately fixed with 2% paraformaldehyde and 2% glutaraldehyde in 0.1M PBS at pH7.4 (on ice, 2 hr). Specimens were then post-fixed with 2% OsO_4 in 0.1M PBS at pH7.4, dehydrated through a graded series of ethanol, and embedded in epoxy resin (Quetol-812, Nisshin EM Co. Ltd., Japan). Ultrathin sections were stained with uranyl acetate and lead hydroxide, and observed with a JEM1010 electron microscope (JEOL Ltd., Japan).

We used wild-type (*Canton S*) and transgenic (*RabRP1 (N601I) / rh1-gal4*) flies for immunoelectron microscopy. Compound eyes of the flies (3 days after eclosion) were fixed with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M Na-Cacodylate buffer (CB, pH7.4) containing 0.05% $CaCl_2$ (on ice, 2hr). After several rinses with CB, specimens were dehydrated through a graded series of ethanol, and then embedded in medium grade LRWhite resin (London Resin Co. Ltd., UK). Ultrathin sections 70 nm thick were picked up on formvar-coated nickel grids, and treated with saturated aqueous solution of sodium meta-periodate for 30 min. After several rinses with distilled water, the specimens were incubated with PBSG (4% BSA, 0.25% gelatin in 0.1 M PBS) at 20°C for 30 min to eliminate the non-specific binding of antibodies. The sections were then incubated overnight with anti-RABRP1 antiserum diluted 1:30 in PBSG at 4°C. After several rinses with PBSG, the sections were incubated with goat anti-mouse IgG conjugated with 15 nm colloidal gold particles (British Biocell, UK) at 20°C for 30 min. Following several rinses with PBSG and subsequently with distilled water, specimens were stained with 4% uranyl acetate and observed with a JEM1010 electron microscope (JEOL Ltd., Japan). For negative controls, anti-RABRP1 antiserum was replaced with preimmune mouse serum.

Generation of transgenic flies

We generated transgenic fries that expressed mutated RabRP1 protein having a dominant negative effect to native RABRP1. The mutant protein was composed solely of the Rab-domain of RABRP1, who's Asn-601 in the third GTP-binding / hydrolysis motif is replaced by isoleucine. In order to substitute Ile for Asn-601 and introduce preferable restriction endonuclease sites at both ends of the cDNA fragment, we performed PCR. We designed a pair of oligonucleotide primers (RP1M-F2, ATCCTGCTCGCGATTAAATGCGAC; RP1M-R1, ACATGAACCTCGAGTGGACGTAG). In the forward primer (RP1M-F2), 3 nucleotide substitutions, TGGCCA → TCGCGA and AAT → ATT, were introduced. Two of them (TGGCCA → TCGCGA) introduced a novel restriction endonuclease (*Nru*I) site without any amino acid substitutions, whereas another (AAT→ATT) caused the substitution of Ile for Asn-601. The reverse RP1M-R1 primer included two substitutions (CTCCAT → CTCGAG), which introduced a *Xho*I site in the 3'-noncoding region of the cDNA. Using these primers, the mutant cDNA fragment was amplified, and digested with *Nru*I and *Xho*I. The wild-type RabRP1 cDNA in pBluescript was digested with *Bal*I and *Xho*I, and the mutant cDNA fragment was inserted into the position. The second PCR was carried out to remove the 5'-noncoding region of mutant RabRP1 cDNA that possibly interferes with the expression of mutant RabRP1. The forward primer (RP1E-F1, CGGAATTCGCATGCCGGCCTTCGGTGACC) was designed at the position including the start point of Rab-domain (Met-456). The primer includes 5 nucleotide substitutions, 4 of them (CCAAGC → GAATTC) generating an *Eco*RI site immediately upstream to the start point of Rab-domain. The last substitution (CCATGC → GCATGC) introduced the *Sph*I site (not utilized in the present study) without any amino-acid substitution. Using the mutant RabRP1 cDNA as a template, we carried out the second PCR with RP1E-F1 and RP1M-R1 primers. The amplified cDNA was then digested with *Eco*RI and *Xho*I, and inserted into pUAST transformation vector DNA.

For transformation, pUAST-RabRP1(N601I) plasmid was purified with a Qiagen plasmid midi kit (Qiagen, Germany), and injected into the eggs of the fly, *B-#1610 (w*; Dr1/TMS, Sb1 P{ry$^{+t7.2}$ =Delta2-3}99B)*. After crossing with wild-type flies (*w1118*), 9 heterozygous insertion lines, each containing a *UAS-RabRP1(N601I)*, were isolated. Females of the transgenic flies were crossed with males carrying the Gal4 gene under control of the *rh1* (*ninaE*) opsin promoter. Then heterozygote flies carrying both *UAS-RabRP1 (N601I)* and *rh1-Gal4* genes were isolated and used for experiments.

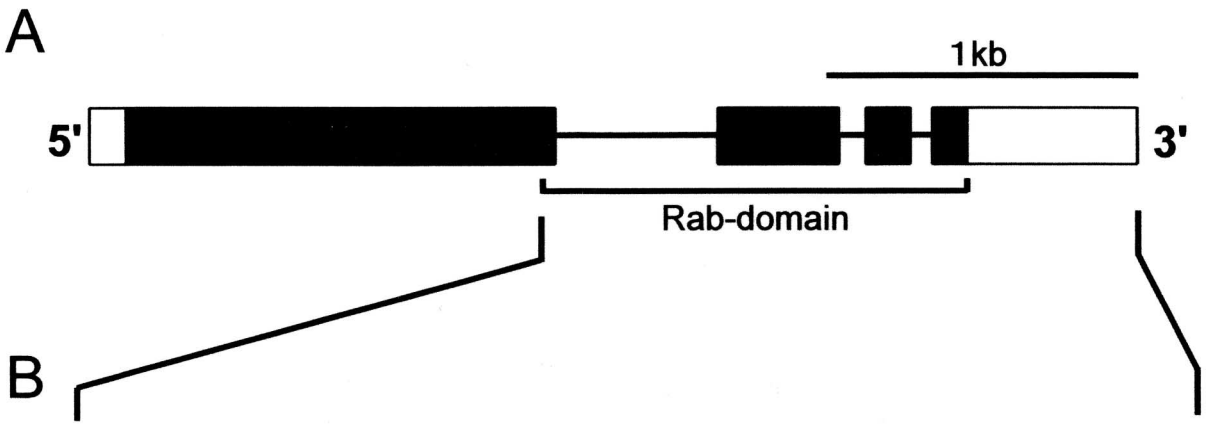
In order to suppress the expression of RABRP1, we generated transgenic flies that expressed the anti-sense RNA of RabRP1 using the Gal4-UAS expression system (Deng *et al*., 1999). cDNA encoding the whole part of the Rab-domain of RABRP1 was inserted into pUAST in the reverse direction. The plasmid was amplified in *E. coli* cells and purified with a Qiagen plasmid midi kit (Qiagen, Germany). Seven heterozygous insertion lines, each containing a *UAS-RabRP1(antisense)*, were isolated using the method described above. Females of the transgenic flies were crossed with males containing the Gal4 gene under the control of the *GMR* regulatory element.

RESULTS

cDNA and deduced amino acid sequence of *Drosophila* RabRP1

A *Drosophila* head cDNA library of 6×10^5 primary clones was screened with the PCR fragment of RabRP1. Several positive clones, each of which carried a different length RabRP1 cDNA fragment, were isolated and sequenced. By comparison with the genomic sequence determined by the *Drosophila* genome project, it was revealed that the longest cDNA fragment (1450bp; GenBank accession number, AB035646) was still incomplete, and composed of the sequence encoding the C-terminal half of RabRP1 and the 3'-noncoding sequence. Therefore, we carried out the 5'-RACE protocol, and determined the full-

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1490 ATGCCGGCCTTCGGTGACCTGACCATGGATCAGGAAATGGAACCGGCCATCATGACATCCACCAGCGATAAGCGC
 456 M  P  A  F  G  D  L  T  M  D  Q  E  M  E  P  A  I  M  T  S  T  S  D  K  R

1565 GAGCATCTGTACAAGATTCTAGTCATTGGTGAACTGGGCACCGGCAGGACGTCCTTCATTAAGCGCTATGTGCAC
 481 E  H  L  Y  K  I  L  V  I  G  E  L  G  T  G  K  T  S  F  I  K  R  Y  V  H
                                 I
1640 CAGTTCTTCAGCCAGAACTACAGGGCCACCATCGGCGTGGACTTCGCCCTGAAAGTGCTGCAGTGGGACGCCAAT
 506 Q  F  F  S  Q  N  Y  R  A  T  I  G  V  D  F  A  L  K  V  L  Q  W  D  A  N
                                    E
1715 ACGATAGTGCGCCTCCAGCTGTGGGATATCGCCGCTCAGGAGAGGTTTGGCAACATGACCCGTGTCTACTACAAG
 531 T  I  V  R  L  Q  L  W  D  I  A  G  Q  E  R  F  G  N  M  T  R  V  Y  Y  K
                              II
1790 GAGGCGGTGGGCGCGTTCATCGTTTTTGATGTGACTCGCAGTGGGACTTTCGATTGCGTGAGCAAGTGGAAGGAA
 556 E  A  V  G  A  F  I  V  F  D  V  T  R  S  G  T  F  D  C  V  S  K  W  K  E

1865 GATCTAGACTCCAAGGTGCAGCTGCCGGACGGCAGTCCCATTCCGTGCATCCTGCTGGCCAATAAATGCGACCAG
 581 D  L  D  S  K  V  Q  L  P  D  G  S  P  I  P  C  I  L  L  A  N  K  C  D  Q
                                                            III
1940 GAAAAGCAGGGCATCATAACGCAGCCCGAGAAGATGGATGAGTATGTGAGGGAGAACGGATTTGCCGGTTGGTTT
 606 E  K  Q  G  I  I  T  Q  P  E  K  M  D  E  Y  V  R  E  N  G  F  A  G  W  F

2015 GAGACATCCGCCAAGGAGAATATCAACATCGATGAGGCAGCTCGTGCCCTCGTGAATAAGATACTCATCAACGAT
 631 E  T  S  A  K  E  N  I  N  I  D  E  A  A  R  A  L  V  N  K  I  L  I  N  D
          IV
2090 AAGCTGATATCTGCCGATCTGGCCGATGGCGACAAGTTCAACTTGAGCGCGGCCGATGCAACCGGATCGGATGCC
 656 K  L  I  S  A  D  L  A  D  G  D  K  F  N  L  S  A  A  D  A  T  G  S  D  A

2165 AAGAACAAGTGTTCCTGCTGACCTGGAACCGAGGTCCGGCGACTCCAATTCCCATTCCCAGTTTAAGAGCGAGTT
 681 K  N  K  C  S  C  *

2240 TCAACACAGAAACGCCATGTGCCTACGTCCACTCCATGTTCATGTTGTTAGAAGATAGCGATAACTATGCTTAGG
2315 GTTTTAACGTAACATTTGTCTAAGACTTAGTGTCATCCGTATCATCCCGTCCGTCTTTATCTTTAACGCCCCGTC
2390 TGTGGGTCCACAATGTTGCGCCGGCATCGGGATCCGTGTCACTTTGAGAATTTATATGGTGACACATAACATACC
2465 TACGCATACGAAACATTATTACTATCACCATCATTGCCCTAGTTGCGAGGATTAGCCGGAGGTAGTGCAGCATCC
2540 GTATCATAGCATACTACATTTGTGTAAAATACGAATCGTCTTATTGTTTTGTTTGTTTTATTTGCCTCTGCTGGC
2615 GAGATTGTTTTATCTTGGAATTATTATTCTCGTATGCAAACACACTCTGCCATCATTATTTGCATTTAGATAACA
2690 ATACGTACGCTTAATGCAATACGAAATCAGAAATAAAGTTTTTAATGGTAGTTAAGTT
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Fig. 1. Cloning and sequencing of RabRP1 cDNA. (A) Genomic organization of the RabRP1 gene. Boxes and lines represent exons and introns, respectively. Open boxes indicate 5'- and 3'-untranslated regions and filled boxes indicate the protein coding region. The amino-acid sequence encoded by the 3'-side of the coding region shows significant homology with Rab-family proteins, and is therefore referred to as the Rab-domain. (B) cDNA and deduced amino acid sequences of RabRP1. The region composed of a Rab-domain and a 3'-untranslated region is demonstrated in particular. A possible polyadenylation signal (AATAAA) is shaded in the nucleotide sequence. The shaded sequences labeled by I–IV are GTP-binding / hydrolysis motifs conserved in the small G-protein superfamily. The sequence labeled by E is the effector region conserved in the Rab-protein family. Cysteine residues in an open box at C-terminal are possible fatty-acid binding sites. Accession number: AB035646.

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length sequence of RabRP1 cDNA. Fig. 1A schematically shows the genomic organization of the RabRP1 gene. The gene is composed of 4 exons and 3 introns, and the cDNA contains a long open reading frame encoding a protein composed of 686 amino acids. The relative molecular mass (*Mr*) of the protein is calculated to be 74.7 k. A possible polyadenylation signal (AATAAA) locates near the 3'-end of the cDNA (Fig. 1B). Because the C-terminal 1/3 of the deduced amino acid sequence (Met-456 - Cys-686, *Mr* = 25.9 k) obviously showed similarity to that of the Rab small G-proteins (see below), we referred to this region as "Rab-domain". In contrast, a larger N-terminal domain of the deduced sequence shared no apparent homology with any other protein so far reported.

The nucleotide and the deduced amino acid sequences of the Rab-domain are shown in Fig. 1B. The Rab-domain contains all four motifs for GTP-binding and hydrolysis conserved in the small G-protein superfamily. In addition, the domain possesses a consensus sequence (TIGXXF) of Rab-specific effector region. However, a unique amino-acid substitution of isoleucine for threonine conserved in most small G-proteins was found in the second motif for GTP-

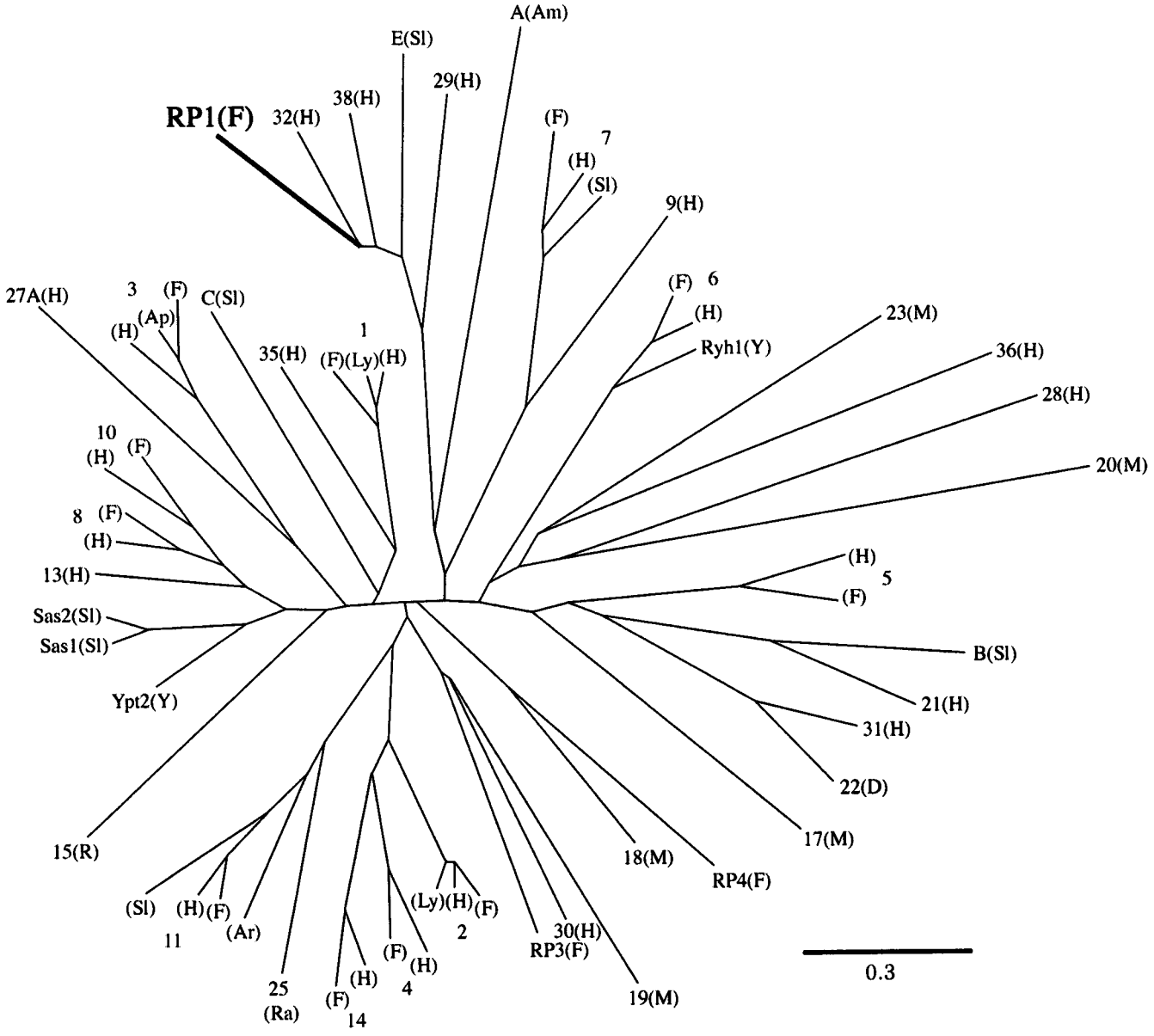


Fig. 2. Phylogenetic analysis of the Rab-family proteins. *Drosophila* RabRP1 (bold) clusters with *Dictyostelium* (Sl; Slime mold) RabE and human (H) Rab32, Rab38 and Rab29. The phylogenetic tree was constructed by the neighbor-joining method using the CLUSTALW program. On all Rab-proteins except RabRP1, full-lengths protein sequences were used for alignment. The sequence of the Rab-domain was used on RabRP1. The abbreviations are; H, human (*Homo sapiens*); M, mouse (*Mus musculus*); R, rat (*Rattus norvegicus*); Ra, rabbit (*Oryctolagus cuniculus*); D, dog (*Canis familiaris*); F, fruit fly (*Drosophila melanogaster*); Sl, slime mold (*Dictyostelium discoideum*); Ly, *Lymnaea stagnalis*; Ap, *Aplysia californica*; Ar, *Arabidopsis thaliana*; Am, Amoeba (*Entamoeba histolytica*); Y, Yeast (*Saccharomyces pombe*). Bar = 0.3 evolutionary distance

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binding and hydrolysis (WDIAGQE). In the carboxyl end of the protein, two cysteine residues were found with a motif of –CXC. This motif, together with –CC and –CCXX, allows lipid modification of both the cysteine residues most likely to have geranylgeranyl prenylation, which is commonly found in the small G-proteins.

We next constructed a molecular phylogenetic tree of Rab family proteins using the Rab-domain of RABRP1. As shown in Fig. 2, RABRP1 is clustered with *Dictyostelium* RabE (AF116859), and mammalian RAB29 (D84488), RAB32 (Q13637) and RAB38 (NM022337). The amino acid identities of RABRP1 with the members of the cluster were between 43–62%, whereas those with other Rab proteins were below 30%.

Fig. 3 shows the sequence alignment of RABRP1 (Rab-domain) together with RabE, RAB29, RAB32, and RAB38. Sequences of some other Rab proteins are also aligned for comparison. In addition to the five conserved motifs, several amino acids widely conserved in the Rab family proteins were also found in RABRP1 (K-485, Y/F-553, K/R-555, D-565, W-578, N-639). Interestingly, the amino acid substitution of isoleucine for threonine in the second motif for GTP binding and hydrolysis (WDTAGQE → WDIAGQE) also occurred in other members of the RabRP1 cluster (RabE, Rab29, Rab32, Rab38). Furthermore, we found another amino acid substitution specific to the members of the RabRP1 cluster; the substitution replacing glycine in the third motif for GTP binding and hydrolysis by alanine (GNKCD → ANKCD). In spite of these substitutions in the conserved motifs, GTP-blot analysis demonstrated that RABRP1 still retained the ability to bind GTP (data not shown).

Tissue-specific distribution of RABRP1

In order to investigate the tissue specificity of RabRP1 gene expression, we carried out northern hybridization for

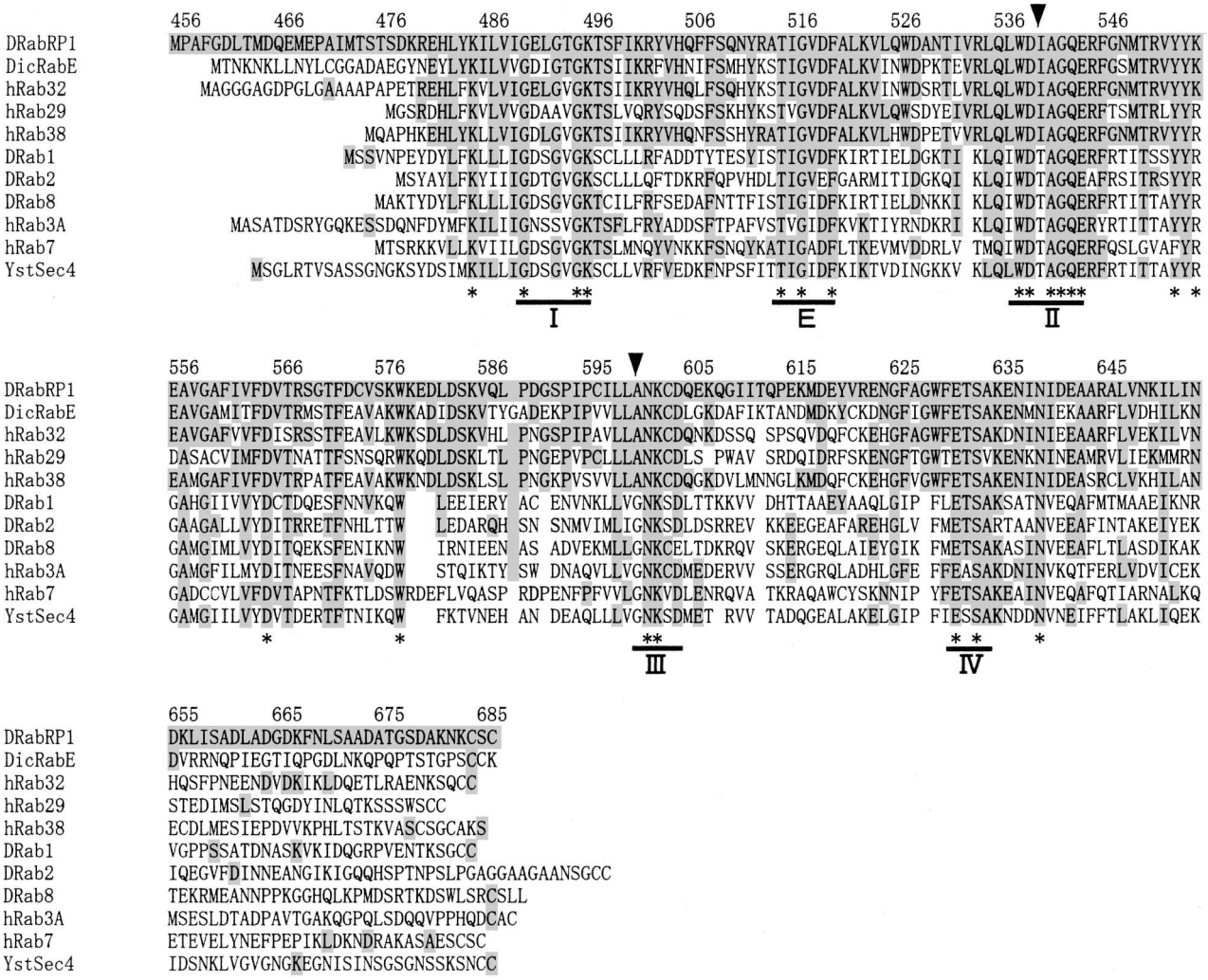


Fig. 3. Comparison of RabRP1 amino acid sequence with other members in the RabRP1 cluster. For comparison, sequences of some other human (h), *Drosophila* (D) and yeast (Yst) Rab proteins are aligned together. The amino acid residues identical to that of RabRP1 are shaded. Asterisks indicate the amino acid residues conserved in most Rab proteins. Arrowheads indicate the positions where amino acid substitution specific to RabRP1 and its relatives are occurring within the conserved motifs I–IV and E.

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mRNAs extracted from several kinds of *Drosophila* tissues. We used a cDNA fragment encoding the complete coding region of Rab-domain of RabRP1 as a probe. As shown in Fig. 4, positive bands at 2.7 kb and 3 kb were detected in ovary and retina, respectively. The lengths of these mRNAs well matched to the total length of RabRP1 cDNA described above (Fig. 1A). On the other hand, a shorter mRNA of 1.5 kb was predominantly identified in testes mRNA, suggesting that RabRP1 mRNA in testis undergoes a different processing from those occurring in retina and ovary.

We next investigated the expression RabRP1 protein (RABRP1) and its tissue-distribution, using an antiserum

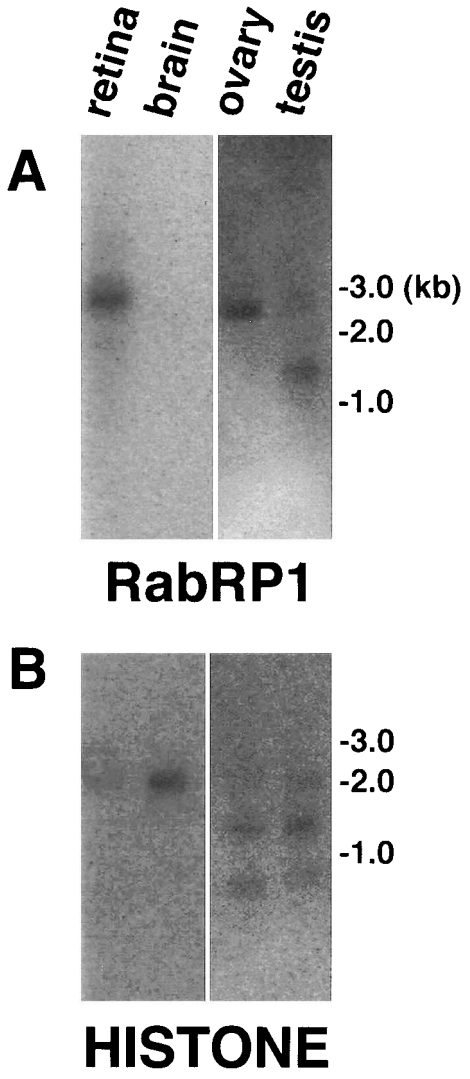


Fig. 4. Northern blot analysis of RabRP1 expression in *Drosophila* tissues. Poly(A)$^+$ RNA, 1000ng from testis and 300-500ng from other parts of wild-type *Drosophila* (50 retinas, 64 brains, 20 ovaries and 180 testes / lane), was electrophoresed and transferred onto a nylon membrane. Blot was initially hybridized with a 1 kb *BamH*I fragment of cDNA including complete Rab-domain of RabRP1 (A). After exposure to X-ray film, the membrane was washed to remove the probe, and rehybridized with histone cDNA probe (control, B).

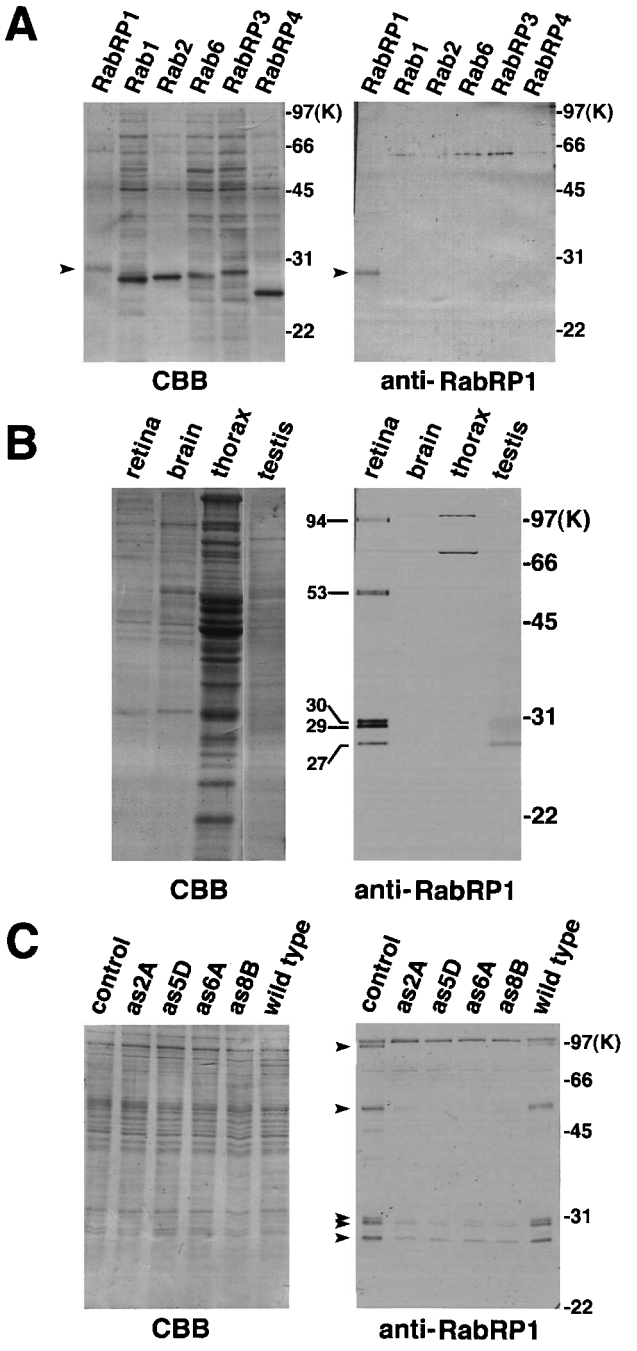


Fig. 5. Immunoblot analysis on the distribution of RABRP1 in *Drosophila* tissues. (A) Antigen specificity of anti-RABRP1 antiserum. Various RAB-6×His and RABRP-6×His fusion proteins were expressed in *E. coli* cells. The total proteins were then separated by SDS-PAGE, blotted onto a PVDF-membrane, and detected with CBB (left) or anti-RABRP1 antiserum (right). Arrowheads; RABRP1. (B) Distribution of RABRP1 in *Drosophila* tissues. In each lane, proteins from a single tissue of wild-type *Drosophila* were electrophoresed. Proteins were then transferred onto a PVDF membrane, and stained with CBB (left) or anti-RABRP1 antiserum (right). (C) Reduced expression of RABRP1 with antisense RNA. Proteins from 2 retinas (CBB staining, left) or a single retina (immunostaining, right) are electrophoresed. Compared with wild-type and GMR-Gal4 control flies, expressions of all immunoreactive peptides (27 k, 29 k, 30 k, 53 k and 94 k, arrowheads) are significantly reduced in 4 different strains that express RabRP1 antisense RNA (as2A, as5D, as6A and as8B).

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against the Rab-domain of RABRP1. Immunoblot analysis demonstrated that the antiserum specifically recognized RABRP1 without any crossreaction with other *Drosophila* Rab proteins (Fig. 5A). Using the antiserum, we examined the expression of RABRP1 in retina, brain, thorax and testis (Fig. 5B). In the retina, the antiserum recognized five clearly separated bands of peptides (27 k, 29 k, 30 k, 53 k and 94 k), among which the 29 k band was most predominant. In addition, all kinds of these peptides were principally recovered in the membrane fraction, when retinas were separated into soluble and membrane fractions (data not shown). This result suggests that the peptides mainly exist in a membrane-bound form. In testis, only smaller bands (27 k, 29 k and 30 k) were detected, and the 27 k band was predominant. No specific signals of anti-RABRP1 were identified in brain and thorax (signals at ~70 k and ~100 k were due to the nonspecific immunoreactions of secondary antibodies), indicating that RABRP1 was exclusively expressed in the retinal and the gonadal organs.

To examine whether the five immunoreactive bands in retina are actual products of the RabRP1 gene, we performed immunoblot analysis on the flies expressing antisense RabRP1 RNA. In these flies, the hybridization of antisense RNA with RabRP1 mRNA suppressed the activity of mRNA, and specifically reduced the yields of the RabRP1 gene products. By germline transformation, we obtained 7 strains of transgenic flies carrying *UAS-RabRP1(antisense)* heterozygously. Four strains of them were used for experiments. As shown in Fig. 5C, the yields of 5 immunoreactive products in the retina were concomitantly reduced, when antisense RNA was expressed with GAL4 under control of the *GMR* regulatory element. This result demonstrated that these 5 peptides were apparently produced from the RabRP1 mRNA. The *Mr* of RABRP1 calculated from the complete sequence of cDNA was 74.7 k, which was significantly smaller than 94 k of the largest immunoreactive peptide. A possible interpretation was that the largest band might arise from oligomerization or post-translational modification of the smaller products.

Cellular and subcellular Localization of RABRP1 in *Drosophila* retina

We immunohistochemically investigated the localization of RABRP1 in *Drosophila* retina. Fig. 6 shows the immunofluorescent staining of a fly head cryosection with anti-RABRP1 antiserum. A fluorescent signal was observed over the entire region of the retina, whereas no signal was detected in the brain (Fig. 6B). This result agreed with the immunoblot analysis (Fig. 5B). Further observation of the retina revealed that strong fluorescence was located immediately beneath the corneal cuticle (Fig. 6B). In detail, the fluorescence exhibited a cup-shaped profile surrounding a pseudocone (Fig. 6C). This pattern of fluorescence staining overlapped with distribution of the retinal pigment cells, suggesting that RABRP1 was predominantly distributed in the cells in the retina.

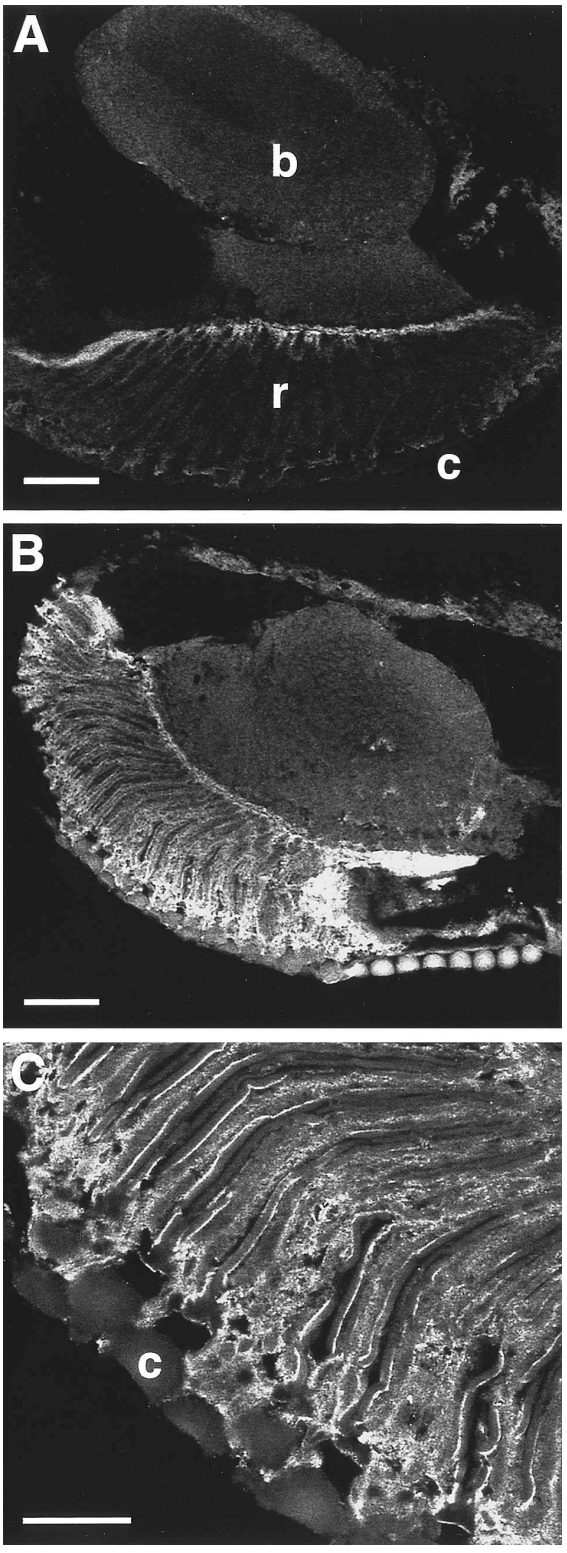


Fig. 6. Immunofluorescence localization of RABRP1 in compound eyes of *Drosophila*. Frozen sections of compound eyes were stained with anti-RABRP1 antiserum, visualized with biotinylated secondary antibody and FITC-conjugated streptavidin, and observed with a confocal laser microscope. (A) Control experiment using preimmune serum for anti-RABRP1 antiserum. Bar, 50 μm. (B) A longitudinal section of the retina was stained with anti-RABRP1 antiserum. Bar, 50 μm. (C) Higher-power magnification image of the retina in (B). Bar, 20 μm. r; retina, b; brain, c; corneal cuticle.

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In the photoreceptor cell layer, many fibrous signals ran along the longitudinal axes of retinal cells, in addition to a diffuse staining over the cell cytoplasm (Fig. 6B). The fibrous signals were localized at the borders between rhabdomeres and photoreceptor cell bodies (Fig. 6C). This staining pattern was reminiscent of the distribution of the pigment granules in photoreceptor cells, as well as that of the subrhabdomeric cisternae (SRC) derived from the smooth endoplasmic reticulum (Matsumoto-Suzuki *et al*., 1989). The results thus suggest that membrane-bound form of RABRP1 was localized at one or both of these organelles in photoreceptor cells.

We further investigated the subcellular localization of RABRP1 in the retina by immunoelectron microscopy (Fig. 7). In cryosections, pigment granules looked like empty vacuoles, because their contents had been eluted out during the fixation processes, while the integumentary membranes of the granules were still reserved. In the photoreceptor cells (Fig. 7A) and the secondary pigment cells (Fig. 7D), colloidal gold particles for RABRP1 were localized on the integumentary membranes of pigment granules. No significant immunoreactivity was detected in the rhabdomere and cytoplasmic parts of the cells. In addition to the control experiments using preimmune sera (Figs. 7B, E), we also performed immunolabeling with an anti-opsin monoclonal antibody. In these experiments, no significant signal was detected on the pigment granules, whereas rhabdomeres of R1-6 photoreceptor cells were densely labeled with anti-opsin antibody

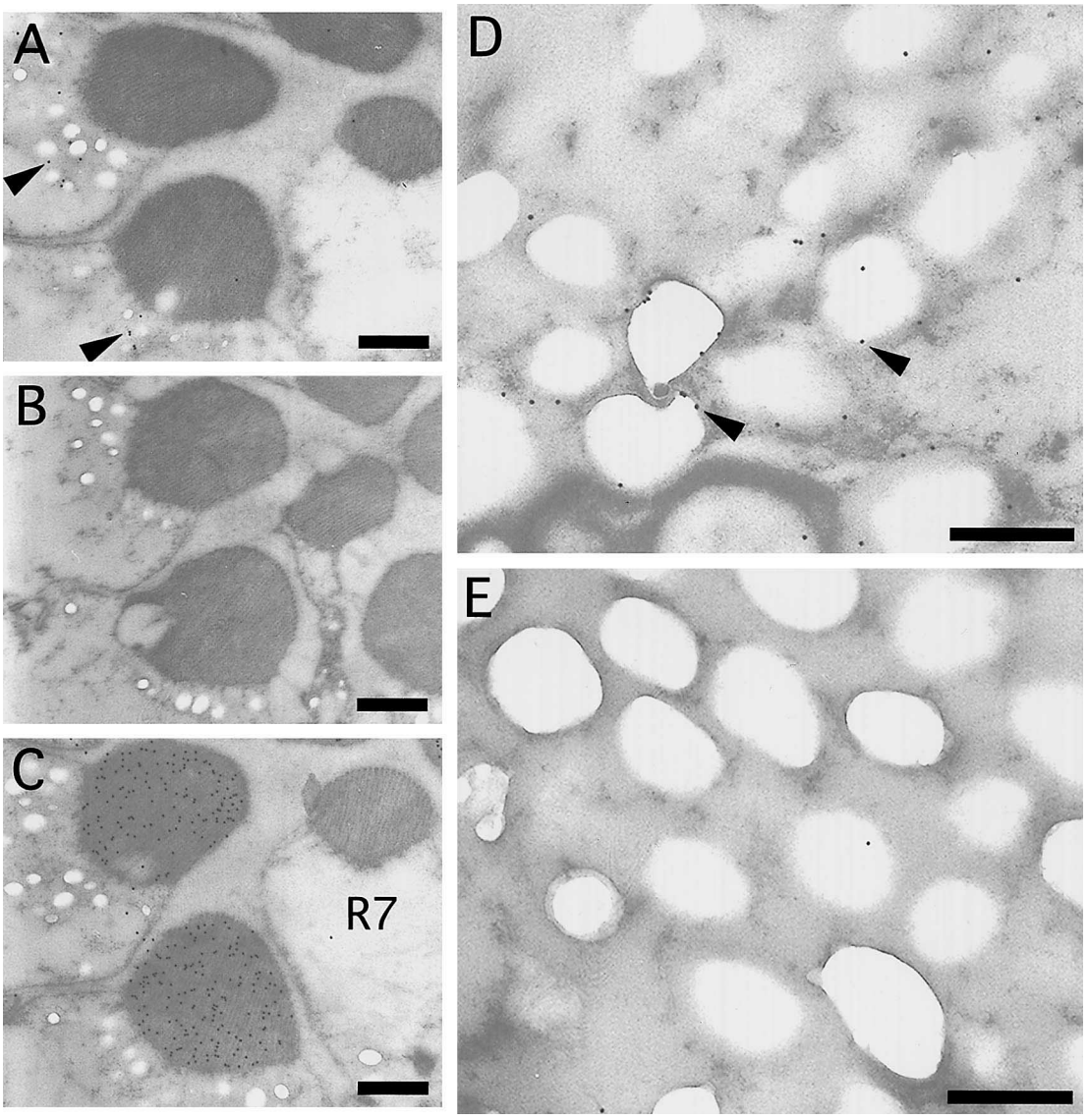


Fig. 7. Immunoelectron micrographs indicating subcellular localization of RABRP1 in *Drosophila* retina. Retinas of wild-type (Canton S) flies were stained with anti-RABRP1 antiserum (A, D). For control experiments, retinas were also stained with preimmune serum (B, E) or anti-Rh1-opsin monoclonal antibody (C). During the preparation of specimens, the content of pigment granules was usually lost, while their boundary membranes remained. Consequently, pigment granules look like empty vacuoles in specimen. RABRP1 signals are found on the rims of pigment granules (arrowheads in A, D). Bar, 500 nm.

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(Fig. 7C). These results thus indicated that RABRP1 was associated with the pigment granules both in photoreceptor and secondary pigment cells.

Accumulation of the autophagosome-like structures in the dominant negative RabRP1 mutant

In order to address the function of RABRP1 *in vivo*, we generated flies that expressed mutant RabRP1 protein exhibiting the dominant inhibitory effect against native RABRP1. In the mutant protein (RABRP1(N601I)), isoleucine was substituted for asparagine in the third GTP-binding and hydrolysis motif. It has been demonstrated that the equivalent substitution of Ile for Asn transforms native RABs to their dominant inhibitors in various kinds of Rab proteins including Rab1 (Satoh *et al*., 1997a; Tisdale *et al*., 1992), Rab5 (Bucci *et al*., 1992) and Rab7 (Feng *et al*., 1995).

The transgenic *GalUAS-RabRP1(N601I)* fly was crossed with the *rh1-Gal4* fly, and their progenies expressing RABRP1(N601I) in R1-6 photoreceptor cells were examined. Fig. 8 shows the electronmicrographs of the photoreceptor cells in the mutant. It is noteworthy that numerous autophagosome-like structures, which look to contain their own cytoplasmic components, could be observed at the bases of rhabdomeric microvilli in the R1-6 photoreceptor cells (Figs. 8A, C). Together, many darkened multivesicular endosomes and large dense granules (300–500nm in diameter), apparently related to the lysosomal pathway, could be observed in the region. Neither R7 photoreceptor cells (Fig.

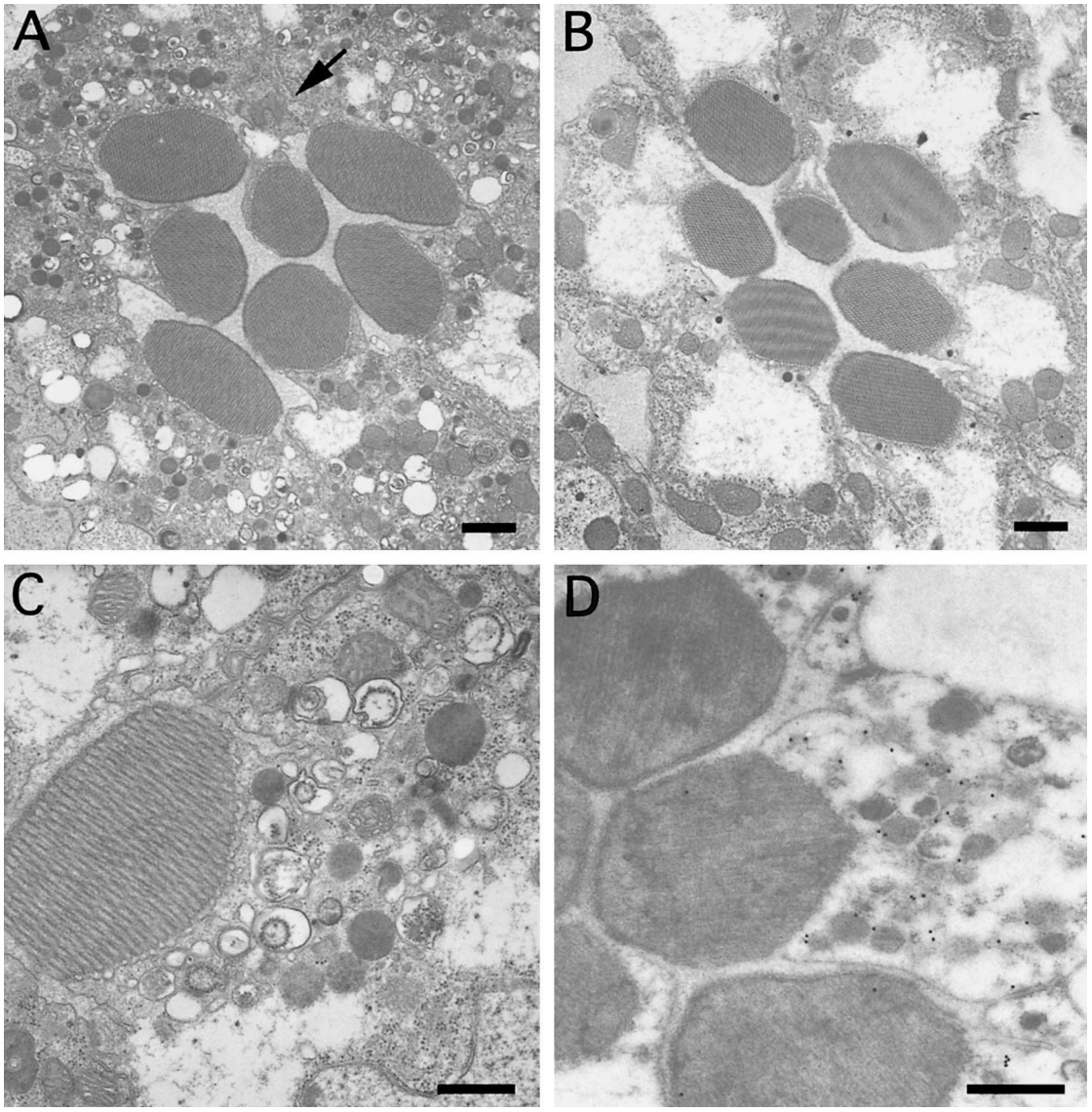


Fig. 8. Morphological defects of photoreceptor cells in *RabRP1 (N601I)* transgenic flies. Under the control of rh1-Gal4, dominant negative protein of RABRP1, RABRP1(N601I), was expressed exclusively in R1-6 photoreceptor cells. (A) Cross-section of a single ommatidium. Numerous electron-dense vesicular/granular structures are abnormally accumulated in the R1-6 photoreceptor cells. Such accumulation is not observed in the R7 photoreceptor cell (arrow). (B) Cross section of control fly possessing an rh1-Gal4 but not a UAS-RABRP1(N601I) gene. (C) High-power magnification image of the photoreceptor cell in *RabRP1(N601I)* transgenic fly. (D) Immnoelectron micrograph indicating the localization of RABRP1(N601I) on the accumulated electron-dense vesicular/granular structures. Bar, 1 μm (A, B), 500 nm (C, D).

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8A, arrow), in which the *rh1* promoter is silent, nor R1-6 cells of the control fly carrying only *rh1-Gal4* gene (Fig. 8B) accumulated such autophagosome-like structures, indicating that the phenotype of the mutant actually resulted from the expression of RABRP1(N601I). Furthermore, immuno-electronmicroscopy revealed that RABRP1(N601I) specifically localized on the autophagosome-like or lysosome-related structures (Fig. 8D). These results thus suggested that RABRP1(N601I) interfered with the degradation of endosomes and autophagosome-like structures through the lysosomal pathway.

DISCUSSION

Unique characteristics of RABRP1

In the present study, we identified a novel Rab protein, RabRP1, in *Drosophila* and investigated its molecular and functional characteristics. Although the primary structure of the protein has been deduced from the genome sequence, the features of the protein actually expressed *in vivo* still remain to be clarified. Here, we demonstrated several distinctive features of RABRP1, which are different from those of other Rab proteins. First, multiple kinds of translational products having different relative molecular masses (94 k, 53 k, 30 k, 29 k and 27 k) were produced from the RabRP1 gene in the eye. This result was confirmed by two different techniques; the inhibition of RABRP1 expression with antisense RNA and immunoblot analysis using antibodies against the Rab-domain of RABRP1. Among these peptides, the 94 k and 53 k peptides were significantly larger than any other Rab proteins so far found whose relative molecular masses are between 20 k and 30 k. However, these large peptides cannot be detected in testes. In this study, we also demonstrated that the large mRNA for RabRP1 (3 kb) having an extraordinarily long open reading frame (ORF) was expressed in the eye, whereas only a much smaller mRNA (1.5 kb) was detected in testes. Furthermore, the Rab-like sequence (Rab-domain, 25.9 k) of RABRP1 was encoded in the 3'-end of 3 kb mRNA of RabRP1. These results suggested that the large peptides in the eye are translational products of the long ORF and thus contain additional sequences to the N-terminal of the Rab-domain. In contrast, 27–30 k peptides would be composed of the Rab-domain alone. Although multiple peptides are expressed in the eye, the major product of the RabRP1 gene was the 29 k peptide. Furthermore, no large peptides were detectable in testes. These results suggest that the Rab-domain is the actually functional domain of RABRP1 *in vivo*. This assumption was strongly supported by the fact that amino-acid substitution in the Rab-domain (RABRP1-N601I) dominantly inhibited the function of RABRP1.

Second, RABRP1 was characterized by its tissue-specific distribution in fly. A lot of the members of the Rab-protein family are involved in general pathways for endocytosis and exocytosis, and are thus distributed ubiquitously in every tissue and organ in the body. For example, RAB1 and RAB5 are involved in the exocytotic process from rER to Golgi body (Satoh *et al*., 1997a) and the endocytic process for early endosome, respectively, and are found ubiquitously in the body (Satoh *et al*., 1998). On the other hand, some kinds of Rab proteins are distributed in a tissue-specific manner. For example, it has been reported that RAB3A and RAB17 are specifically distributed in neural (Fischer von Mollard *et al*., 1990) and epithelial (Lutcke *et al*., 1993) tissues, respectively. It should be noted that such tissue-specific distributions of Rab proteins are usually associated with their specific functions in cells. For example, RAB3A functions in the fusion process of synaptic vesicles. In this study, we demonstrated that RABRP1 was predominantly expressed in the eye and testis unlike any other Rab proteins so far reported. This result therefore suggested that RABRP1 possibly plays a unique role in these tissues. Details on the RABRP1 function are discussed later.

Third, the Rab-domain of RABRP1 included unique amino-acid substitutions within the conserved GTP-binding/hydrolysis motifs of the protein (Ile-540 and Ala-600). Despite these substitutions in the conserved motifs, RABRP1 still retained the ability to bind GTP. However, this result do not exclude the possibility that there may exist significant difference in enzyme kinetics between RABRP1 and conventional Rab proteins. Furthermore, the equivalent substitutions were also found in mammalian Rab32, Rab38, Rab29 and *Dictyostelium* RabE proteins whose functions remain to be elucidated. The phylogenetic analysis of Rab proteins indicated that RabRP1, together with these 4 proteins, formed a cluster in the phylogenetic tree. Interestingly, the recently issued human genomic DNA sequence suggests that human RAB32 also has an extended N-terminal region to give a long peptide with a relative molecular mass of 53 k, although its deduced N-terminal sequence shows no apparent similarity to that of RABRP1. These results suggest that RABRP1 shares functional similarities with mammalian and *Dictyostelium* Rab proteins, and possibly regulates a common process of vesicle transport.

A possible function of RABRP1

In order to elucidate the function of RABRP1, we investigated the cellular and subcellular localizations of the protein and the effects of functional inhibition of RABRP1 by expressing the dominant negative form of the protein (RABRP1N601I). Immunofluorescence microscopy of the compound eye demonstrated that RABRP1 was predominantly localized in the pigment cells just beneath the corneal cuticle. In *Drosophila*, pigment cells having pigment-containing organelles (pigment granules) in the cytoplasm existed exclusively in compound eyes, ocelli and testes. The result indicating the histochemical localization of RABRP1 in the pigment cells thus coincided with that of the immunoblot analysis that demonstrated RABRP1 localizing in the eye and testes of fly. We also investigated the subcellular localization of RABRP1 using immunoelectron microscopy. The results revealed that RABRP1 was localized on the bound-

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ary membranes of pigment granules in the retinal pigment cells and the photoreceptor cells, suggesting that RABRP1 possibly functioned in the vesicle transport pathway involved in the formation or degradation of pigment granules. For functional analysis of RABRP1, we further investigated the morphological defects of photoreceptor cells caused by the overexpression of RABRP1N601I. The results indicated that functional inhibition of RABRP1 with RABRP1N601I induced the abnormal accumulation of autophagosome-like structures in the cells. In addition, many electron-dense multivesicular endosomes and large dense granules, which are apparently related to lysosomal pathway, were also observed in cells. The autophagosome is a cell organelle participating in the degradation of the endogenous proteins and organelles through the fusion with lysosome. These data therefore suggest that RABRP1 functions in the vesicle transport pathway toward lysosomes and/or its related structures. Recently, in mammalian pigment cells (melanocytes), it has been suggested that melanosomes, organelles containing the melanin pigment, are specialized forms of late endosomes or lysosomes, and are formed through a similar pathway to that of lysosomes (Jimbow *et al*., 1997; Schraermeyer and Stieve, 1994; Vijayasaradhi *et al*., 1995). Furthermore, in *Drosophila*, several kinds of proteins required for vesicle traffic to lysosomes are also essential for biogenesis of pigment granules: *garnet* gene product, which functions in the delivery of proteins to pigment granules, was identified as the δ-subunit of the AP-3 adaptor complex that was involved in the Golgi-to-lysosome pathway (Cowles *et al*., 1997; Le Borgne *et al*., 1998; Ooi *et al*., 1997; Simpson *et al*., 1997). The *deep orange* and *light*, genes required for the biogenesis of pigment granules, encode *Drosophila* homologues of the yeast VPS18 and VPS41 gene products, respectively. In the yeast, these proteins are essential for the normal delivery of proteins to the vacuole, the yeast equivalent of the mammalian lysosome (Sevrioukov *et al*., 1999; Warner *et al*., 1998). Based on these data, it is now assumed that the biogenesis of pigment containing organelles (melanosomes and pigment granules) shares common molecular machineries with the protein transport to lysosomes. In the present study, we demonstrated that RABRP1 was localized on pigment granules, and was possibly involved in the lysosomal pathway, too. This result therefore supports the above assumption and suggests that RABRP1 participates in the vesicle traffics for pigment granules using the endosomal / lysosomal pathway. From this point of view, it is very interesting that Rab38, which belongs to the RabRP1 cluster in the Rab-phylogenetic tree, is exclusively localized in cultured melanocytes (Jager *et al*., 2000). In addition, Rab7, a member of the subfamily neighboring on the RabRP1 cluster, is also localized on melanosomes (Gomez *et al*., 2001). These results suggest that RABRP1 and its relatives function in the vesicle transport pathways for pigment-containing organelles, both in insects and mammals.

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