Rab proteins of *Drosophila melanogaster*: Novel members of the Rab-protein family

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Abstract From a *Drosophila* head cDNA library, we isolated 9 cDNA clones, each of which encodes a different member of Rab-protein family. Seven of them (DRabs) have more than 80% amino acid identity to the corresponding members of mammalian Rab proteins. The other two proteins (DRabRP3 and 4) show low sequence similarity to any of the known Rab proteins. However, both contain all amino acids conserved in known Rabs. In addition, DRabRP4 has strong GTP-binding activity, when synthesized in *E. coli* cells. These results indicate that DRabRPs are novel members of the Rab-protein family. Molecular phylogenetic analysis also supported this conclusion.

Key words: Rab; Ras; Vesicle transport; Small G-protein; Phylogenetic tree; (*Drosophila melanogaster*)

1. Introduction

Members of the Rab-protein family of small GTP-binding proteins are required in the control of vesicular transport in the exocytotic, endocytotic, and transcytotic pathways [1,2]. More than 30 different Rab protein have been identified, and some of them are characterized by their distinctive localization in the cell [1]. Each Rab protein is thought to work at a particular stage of a vesicular transport pathway. For example, Rab1 (Ypt1p in yeast) and Rab2 are required for vesicular transport from the rough endoplasmic reticulum (rER) to the Golgi stack [3–6]. Rab6 is distributed from medial Golgi to the trans-Golgi network [7,8]. Rab8 is localized to the trans-Golgi network, post-Golgi vesicles and plasma membrane [9]. Also in endocytosis, Rab proteins seem to perform their functions. Rab4, Rab5 and Rab9 are each thought to be involved in a particular step in this pathway [10–12]. The role of Rab3A has also eagerly been investigated, and is believed to work in the exocytosis of synaptic vesicles [13,14].

The body of evidence showing Rab proteins are implicated in the control of vesicular transport is rapidly growing. However, most studies has been carried out in isolated cells like yeast and mammalian cultured cells. In invertebrates, few Rabs have been identified and cloned [15,16], neither have comprehensive studies been carried out. *Drosophila* is one of the most important experimental animals, the molecular genetics of which have been highly developed. In addition, the use of *Drosophila* is of great advantage to observe differential expression of Rab proteins in various tissue and developmental stages, as well as under different conditions of external environment (light, nutrition, etc.) and internal bioclock. In the present study, we carried out cDNA cloning and characterization of *Drosophila* Rab proteins in order to establish the bases for further molecular and cell biological studies.

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Abbreviations: CBB, Coomassie brilliant blue; DRab, *Drosophila* Rab protein; DRabRP, *Drosophila* Rab-related protein; IPTG, isopropyl β-D-thiogalactopyranoside; PCR, polymerase chain reaction; PVDF, polyvinylidene difluoride; rER, rough endoplasmic reticulum.

2. Materials and methods

All experiments were carried out on white-eyed (*w*) *Drosophila melanogaster*. Flies were reared on a carotenoid-rich medium containing 6% yellow cornmeal, 5% dry yeast, 3.2% sucrose, 0.32% methyl *p*-hydroxybenzoate, and 2% agar. Flies were raised in a room kept at 25°C with a 12 h light/12 h dark cycle of fluorescent lighting at an intensity of 50 lux.

For RT-PCR, poly(A)$^+$ RNA was extracted from dehydrated tissues of the flies. Flies (4–8 days after eclosion) were rapidly frozen in liquid nitrogen and separated into heads and bodies. Acetone at −30°C was added to them and kept at −30°C for 10 days to substitute acetone for water. Acetone was then evaporated at room temperature and the dried heads were further dissected under a microscope to separate retina from optic lobes and brain [17]. Poly(A)$^+$ RNA was directly extracted from the dried retinas of 100 flies by the guanidium thiocyanate method [18], combined with purification employing oligo(dT)-cellulose using a QuickPrep Micro mRNA Purification Kit (Pharmacia). Single-stranded cDNA (ss-cDNA) was made by reverse transcription using oligo $d(T)_{12-18}$ primer [19]. Forward and reverse primers for PCR were designed to amplify the DNA fragments coding the polypeptide between the effector and the third GTP-binding/GTPase-catalytic regions of the Rab-family proteins (Figs. 1A and 2). PCR was carried out between these primers using the above ss-cDNA as the template DNA. The amplified DNA fragments of approx. 270 bp were then recovered, subcloned in pUC18 vector, and sequenced.

In order to construct a *Drosophila* head cDNA library, total RNA was extracted from 5000 frozen heads of flies according to the method of Chomczynski and Sacchi [20]. Poly(A)$^+$ RNA was isolated from total RNA with oligo(dT)-cellulose using a mRNA Separator Kit (Clontech). After the synthesis of double-strand cDNA, adaptor ligation, phosphorylation, and the removal of the short strands ($<$ approx. 200 bp) of cDNA with a MicroSpin S-400 column (Pharmacia), cDNA was ligated with Lambda ZAP II vector arms (Stratagene), and subjected to in vitro packaging reactions using a Gigapack II Gold Packaging Kit (Stratagene). The resultant cDNA library contained 1×10^6 independent recombinants (2×10^6 pfu, 50% recombinant), and was immediately amplified to give the total pfu of 3×10^{11}. Screening of the cDNA library was carried out by plaque hybridization, according to Maniatis et al. [21]. Cloned PCR fragments of DRab/DRabRPs were used for probes. Both strands of the cDNA inserts of positive clones were sequenced by the dideoxy chain termination method using a Taq Dye Primer Cycle Sequencing Kit and a 373A DNA sequencer (Applied Biosystems). The Clustal W program was used for a multiple alignment of amino acid sequences. A phylogenetic tree was constructed by the neighbor-joining method [22].

DRab/DRabRPs having 6×His tags at their C-terminals were expressed in *E. coli* cells. In all cases except DRabRP3, complete coding regions of their cDNAs were amplified by PCR, and introduced into pQE-60 or pQE-70 expression vector (Qiagen). Transformed *E. coli* cells (JM109 or XL1-Blue) were incubated overnight in 2×YT me-

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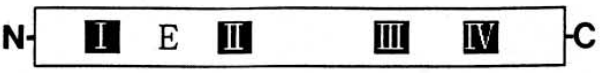
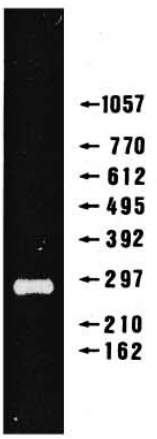
A



Rab F1 primer: T・V/I・G・V/I・D/E・F
5'-GCGAATTC AC(ACGT)(AG)T(ACGT)GG(ACGT)
(AG)T(ACGT)GA(TCAG)TT-3'

Rab R1 primer: G・N・K・C/S/K/T・D・M/C/L
5'-GCGGATCC (AC)(AGT)(AC)TC(ACGT)(TCG)
(AT)(CT)TT(AT)TT(ACGT)CC-3'

B



C

Mammalian RAB

Drosophila RAB	1	2A	3D	4B	6	7A	8	10	11	14
DRAB 1	***95***	54	53	52	40	37	65	62	56	50
2	54	***97***	46	67	41	41	51	51	58	70
3	53	48	***87***	54	36	41	53	51	43	44
4	56	68	50	***82***	45	46	49	47	53	68
6	40	43	36	42	***96***	43	40	44	34	37
7	42	39	40	44	46	***91***	42	47	38	43
8	66	50	51	47	40	42	***91***	78	51	46
10	64	51	51	49	42	42	82	***93***	47	46
11	56	55	50	58	32	40	53	50	***93***	55
14	50	69	42	69	37	47	51	49	55	***92***
DRABRP1	44	41	39	41	43	37	47	35	42	54
2	59	51	49	51	39	43	53	59	47	50
3	59	60	52	52	49	40	52	53	54	57
4	42	44	35	51	37	40	37	42	43	40

Fig. 1. Identification of cDNA fragments for *Drosophila* rab-family proteins (DRab and DRabRP). (A) Synthetic oligonucleotide primers for PCR amplification of DRab/DRabRPs. Degenerated forward (RabF1) and reverse (RabR1) primers were designed from the amino acid sequences for Regions E and III of known rab proteins, respectively. Regions I–IV are conserved in all members of the small GTP-binding protein superfamily and are essential for GTP-binding and GTPase activity of the protein. Region E is the effector region specifically conserved within rab-family proteins. (B) Amplified DRab/DRabRP cDNA fragments (approx. 270 bp) separated by agarose gel electrophoresis. The numbers on the right indicate the positions and sizes (base pairs) of DNA size markers. (C) Percent identities of amino acid sequences deduced from the nucleotide sequences of DRab/DRabRP cDNA fragments to those of the corresponding position of mammalian Rab proteins. Sequences of mammalian rab proteins were obtained from GenBank database (Rab3D: *Mus musculus*, Rab6: *Homo sapiens*, Rab14: *Rattus norvegicus*, others: *Canis familiaris*).

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                                    *   ***                 *** *
HRab1              MSSMNPEYDYLFKLLLIGDSGVGKSCLLLRFADDTYTESYISTIGVDFKI
DRab1              MSSVNPEYDYLFKLLLIGDSGVGKSCLLLRFADDTYTESYISTIGVDFKI
HRab2                  MAYAYLFKYIIIGDTGVGKSCLLLQFTDKRFQPVHDLTIGVEFGA
DRab2                  MSYAYLFKYIIIGDTGVGKSCLLLQFTDKRFQPVHDLTIGVEFGA
DRabRP3   MTARNPQTLMALPNEEHFDFLFKIVLIGDCGTGKTCIVDRFKTGNYIERHGNTIGVDFSM
DRabRP4                 MADRAIKLLVIGESGVGKSSLIRRFVENKFDQNHDVTIGMDFKS
                                       I                   E

                   ***** *        **      **          *
HRab1     RTIELDGKTIKLQIWDTAGQERFRTITSSYYRGAHGIIVVYDVTDQESFNNVKQWLQEID
DRab1     RTIELDGKTIKLQIWDTAGQERFRTITSSYYRGAHGIIVVYDCTDQESFNNVKQWLEEIE
HRab2     RMITIDGKQIKLQIWDTAGQESFRSITRSYYRGAAGALLVYDITRRDTFNHLTTWLEDAR
DRab2     RMITIDGKQIKLQIWDTAGQEAFRSITRSYYRGAAGALLVYDITRRETFNHLTTWLEDAR
DRabRP3   KTIAVEGKQIKLQIWDTAGQERFRTITQSYYRSANGVLIVYDITKRSSFSNLQKWIEEVR
DRabRP4   KVMQVDGIDYKVALWDTAGAERFRSLTPSFYRKALGAILVYDITSRDSLVKLETWLAELD
                          II

                 *** *                      ****      *
HRab1     RYASENVN-KLLVGNKCDLTTKKVVDYTT--AKEFADSLGIPFLETSAKNATNVEQSFMT
DRab1     RYACENVN-KLLVGNKSDLTTKKVVDHTT--AAEYAAQLGIPFLETSAKSATNVEQAFMT
HRab2     QHSNSNMV-IMLIGNKSDLESRREVKKEE--GEAFAREHGLIFMETSAKTASNVEEAFIN
DRab2     QHSNSNMV-IMLIGNKSDLDSRREVKKEE--GEAFAREHGLVFMETSARTAANVEEAFIN
DRabRP3   RYTASNVL-IILVGNKCDLEEQREVDFEEARQMCQYIPEILFVMETSAKENMNVEDAFRC
DRabRP4   SYSDNPNIAIIVVGNKIDEE--RVVDREE--GRKFARKHRALFIETSAKCDQFVSDVFKD
                        III                        IV

HRab1     MAAEIKKRMGPGATAGGAEKSNVKIQSTPVKQSGGGCC
DRab1     MAAEIKNRVGPPSSATDNASKVKIDQGRPVENTKSGCC
HRab2     TAKEIYEKIQEGVFDINNEANGIKIGPQHAATNATHAGNQGGQQAGGGCC
DRab2     TAKEIYEKIQEGVFDINNEANGIKIGQQHSPTNPSLPGAGGAAGAANSGCC
DRabRP3   LANELKRQHDANNVEEVPENTITLGQGKPLKSCSSSCNLT
DRabRP4   VVEKIVSSEYFNNGNASAGLDIASDRDLEASASTCYC
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Fig. 2. Comparison of deduced amino acid sequences of DRab1, DRab2, DRabRP3 and DRabRP4 with human Rab1 (HRab1) and Rab2 (HRab2). Amino acids conserved in most rab proteins are indicated by asterisks. Dotted areas indicate the regions I–IV and region E shown in Fig. 1A. Cys-containing consensus sequences (underlined) for binding of geranylgeranyl groups are found at the C-terminal in all DRabs and DRabRPs.

dium. Expression of tagged proteins was then induced by adding 1 mM isopropyl β-D-thiogalactopyranoside (IPTG), followed by additional incubation for 3 h. The cDNA starting from the second ATG in the reading frame was used in DRabRP3, because the elimination of N-terminal peptide (9 amino acid residues) dramatically increased the yield of DRabRP3. Proteins extracted from the transformed *E. coli* cells were separated by electrophoresis in 12.5% SDS-polyacrylamide gel as described by Laemmli [23]. Proteins were then blotted onto polyvinylidene difluoride (PVDF) membrane using an SDS-containing buffer (0.02% SDS, 100 mM Tris, 192 mM glycine, 15% methanol). The membrane was incubated in renaturation buffer (140 mM NaCl, 3 mM KCl, 3 mM KH_2PO_4, 140 mM Na_2HPO_4, 0.5% Tween 20) for 45 min, followed by incubation in GTP-binding buffer (20 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 0.1% Triton X-100, 74 kBq/ml [α-^{32}P]GTP (29.6 TBq/mmol)) for 2 h. The membrane was then washed 3 times with renaturation buffer, dried on filter paper, and exposed to X-ray film (X-Omat AR, Kodak).

Polytene chromosome squashes were prepared for in situ hybridization as previously described [24]. DNA probes were labeled with digoxigenin/dUTP, which was then detected using a DIG nucleic acid detection kit (Boehringer Mannheim).

3. Results and discussion

In primary structure, every member of Rab protein contains 5 characteristic regions [25]. Four of these regions, forming the GTP-binding and/or GTPase catalytic site (Figs. 1A and 2, I–IV), are conserved in all members of the small G-protein (SMG) superfamily. The fifth is termed an effector site which contains the amino acids especially conserved in Rab-family proteins (Figs. 1A and 2E). We thus made forward and reverse primers for the polymerase chain reaction (PCR) within the effector and the third GTPase related sites, respectively (Fig. 1A). Using the mRNA extracted from *Drosophila* eyes, we carried out RT-PCR to obtain the cDNA fragments of *Drosophila* Rab-family proteins. The amplified cDNA formed

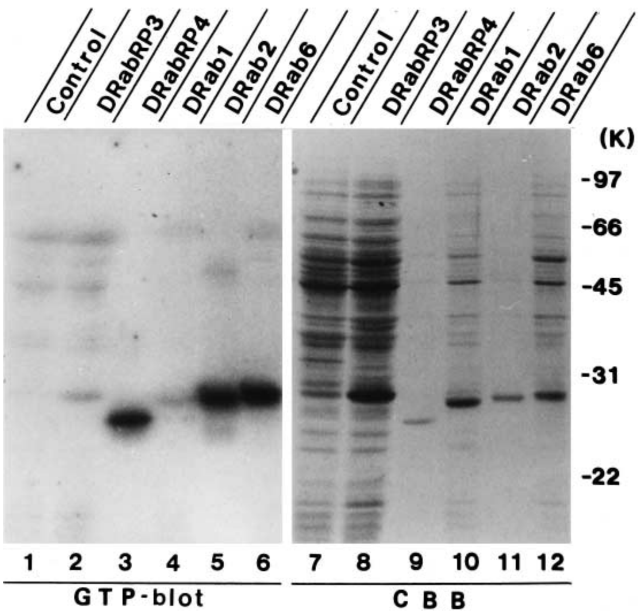

Fig. 3. GTP blotting of DRab1, DRab2, DRab6, DRabRP3 and DRabRP4 expressed in *E. coli* cells. Transformed *E. coli* cells were incubated overnight, followed by the additional incubation with IPTG for 3 h. Proteins were extracted from the cells with SDS-containing buffer, separated by SDS-PAGE, and were blotted onto PVDF membrane. After the binding reaction with [α-^{32}P]GTP, the membrane was subjected to autofluorography (lanes 1–6) followed by the Coomassie Brilliant Blue R-250 staining (lanes 7–12). DRabRP4, as well as DRabs, binds labeled GTP, although the GTP-binding activity in DRabRP3 is hardly detected. Also note that the binding activity in DRab1 is significantly lower than that in DRab2, DRab6 and DRabRP4. The amount of protein charged on each lane was determined so as to show the difference in GTP-binding activity clearly. In control, *E. coli* was transformed with the vector carrying no cDNA inserts, (lanes 1,7). The numbers and the small bars on the right indicate the positions and the sizes of protein size markers.

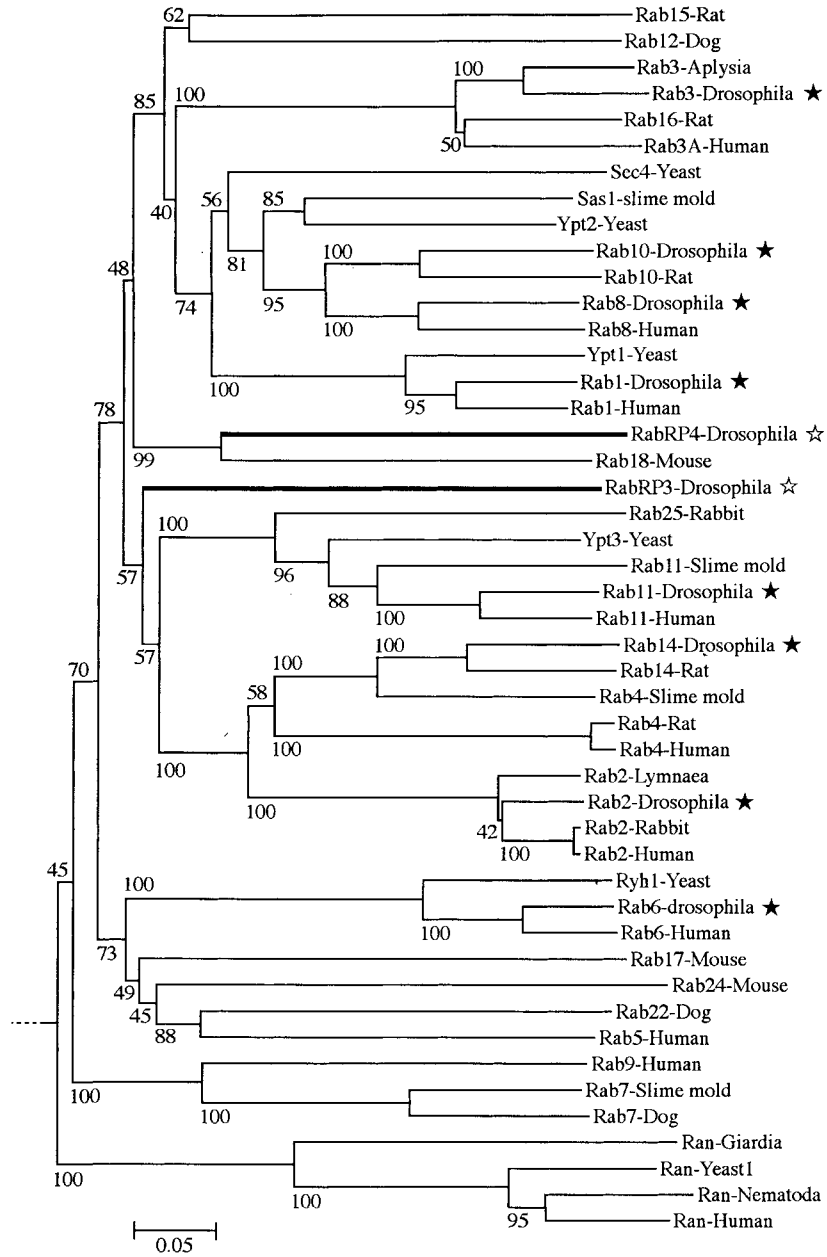

Fig. 4. Phylogenetic tree of Rab proteins constructed by the neighbor-joining method. The deepest root of the tree was determined by constructing another expanded tree that included several Ras proteins as outgroup. Figures on the horizontal lines indicate the bootstrap probabilities (%). All DRabs (closed stars) are included in the known sub-groups of Rab proteins, while DRabRPs (open stars) make new branches near the root of Rab evolution. It is also clear that DRabRPs are included in Rab-protein family which diverged from Ran-protein family. All sequence data except DRabs and DRabRPs were obtained from GenBank. Bar = 0.05 evolutionary distance.

a single band at about 270 bp in agarose gel electrophoresis (Fig. 1B). These cDNA fragments were then cloned and sequenced. In total, 14 different clones were isolated. We compared the amino acid sequences deduced from these cDNA with those of known Rab-family proteins. As shown in Fig. 1C, 10 of these clones (DRabs) showed extremely high amino acid identity to those of mammalian Rab1, Rab2, Rab3, Rab4, Rab6, Rab7, Rab8, Rab10, Rab11 and Rab14, respectively. However, the other 4 clones (DRabRPs) showed a low score of identity to the known Rab proteins.

By screening a *Drosophila* head cDNA library using probes of the above PCR fragments, we isolated complete cDNA clones of DRab1 (D84312), DRab2 (D84313), DRab6 (D84314), DRab8 (D84347), DRab10, DRab11 (D84315), DRab14 (D84316), DRabRP3 (D84348) and DRabRP4 (D84317). As expected from their partial sequences deduced from the PCR fragments, the complete amino acid sequences of DRabs also showed high amino acid identity (> 80%) to those of corresponding mammalian Rabs. On the other hand, the sequences of DRabRP3 and DRabRP4 were again showed low identity (< 40%) to other Rab proteins. To examine whether DRabRP3 and DRabRP4 really belong to the Rab family, we checked their primary structures more closely. In Fig. 2, the deduced amino acid sequences of DRab1, DRab2, DRabRP3 and DRabRP4 are compared with those of human Rab1 and Rab2 proteins. Both DRabRP3 and DRabRP4

contained all amino acids conserved not only in all members of the small G-proteins, but also within the Rab-family proteins. These amino acids are mainly localized in the GTP-binding/GTPase and the effector regions. Moreover, DRabRPs contain the C-terminal consensus sequences (-CNLT and -CYC), where cysteine residues bind with fatty acids. Most small G-proteins have C-terminal cysteine residues, which, by binding with fatty acids, play an essential role in the interaction with other membrane or protein components [26,27]. Therefore, it is likely that DRabRPs would also undergo this modification to play their roles in the cell.

We further examined if DRabs and DRabRPs actually have GTP-binding activity by the GTP blotting method using the proteins expressed in *E. coli* cells. As shown in Fig. 3, all of the DRabs and DRabPR4 were clearly labeled with radioactive GTP. This indicates that not only DRabs but also DRabPR4 are renatured and bind GTP on the blotting membrane as reported in other mammalian and yeast Rab proteins. This result strongly suggests that DRabs and DRabRP4 are functional members of the Rab-protein family serving for the vesicular transport in the *Drosophila* cells. In contrast to DRabRP4, significant binding of GTP was hardly observed in DRabRP3. Since the amino acids required for GTP binding are completely conserved in DRabRP3, renaturation of DRabRP3 on PVDF membrane may be less efficient than that of other DRabs. Otherwise, the GTP-binding activity of DRabRP3 may be very much lower than that of the other DRabs, such a difference in GTP-binding activity reflecting the functional specialization of each Rab protein.

The above characteristics suggest that DRabRP3 and DRabRP4 are novel members of the Rab-protein family. In order to confirm this assumption, we constructed a phylogenetic tree of Rab proteins. As shown in Fig. 4, DRabs (closed stars) are obviously included in the corresponding known Rab subgroups. In contrast, each of the DRabRPs (open stars) diverges from the other Rab subgroups in the early stage of Rab evolution. However, it should be noted that these branchings occurred significantly after the diversion of Rab family from the nearest protein family, Ran. This result supports the above assumption that DRabRP3 and DRabRP4 are novel members of Rab-protein family.

In the pathway of vesicle transport, multiple kinds of Rab proteins are thought to function in cooperative ways to complete the transport from rER to the target membrane. Since this suggests the possibility that genes for those Rab proteins could be present as a gene complex to ensure their cooperative expression, we determined the gene loci of DRab/DRabRPs using in situ hybridization on polytene chromosome. The results, however, indicated no colocalization of any DRab/DRabRP genes on the chromosome (DRab1:93D1-5, DRab2:42C, DRab6:33B9-12, DRab11:93B8-13, D-Rab14:36A-B, DRabRP3:66B-C, DRabRP4:5A8-11). This suggests that the correlational transcription of Rab genes may not contribute to the functional regulation of Rab proteins.

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