Rab6 Regulation of Rhodopsin Transport in Drosophila*

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Rab6 is a GTP binding protein that regulates vesicular trafficking within the Golgi and post-Golgi compartments. We overexpressed wild-type, a GTPase defective (Q71L), and a guanine nucleotide binding defective (N125I) Rab6 protein in Drosophila photoreceptors to assess the in vivo role of Rab6 in the trafficking of rhodopsin and other proteins. Expression of Drab6Q71L greatly reduced the steady state levels of two rhodopsins, Rh1 and Rh3, whereas Drab6N125I showed weaker effects. Analysis of a strain carrying Rh1 rhodopsin under a heat shock promoter showed that Drab6Q71L, but not Drab6N125I or Drab6N125I, prevents the maturation of rhodopsin beyond an immature 40 kDa form. Drab6Q71L is a GTPase defective mutant, indicating that anterograde transport of rhodopsin requires Rab6 GTPase function. The three Drab6 strains had no effect on the expression of several other photoreceptor proteins. The Drab6Q71L photoreceptors show marked histological defects at young ages and degenerate over a two week time span. These results establish that rhodopsin is transported via a Rab6 regulated pathway and that defects in trafficking pathways lead to retinal degeneration.

Members of the Rab family of small GTPases are localized in distinct subcellular compartments (1), and within these compartments they regulate vesicular trafficking by cycling between GTP- and GDP-bound forms (2). A general model of Rab function has emerged in which a complex of Rab-GDP and guanine nucleotide dissociation inhibitor (GDI) is maintained in the cytosol. On binding of this complex to the donor membrane, GDI is displaced and GDP is exchanged for GTP. Rab-GTP is recruited onto the transport vesicle, which buds from the donor membrane and then associates with the target membrane. The Rab-GTP is thought to mediate fusion of the vesicle through interactions with effector molecules on the target membrane. It is not known whether GTP hydrolysis of the Rab-GTP is required for vesicle fusion or occurs after fusion. After GTP hydrolysis, Rab-GDP is retrieved from the target membrane by GDI and recycled to the donor membrane (3–5).

The study of point mutations in several rab genes affecting amino acids essential for guanine nucleotide interactions has documented the importance of the Rab-guanine nucleotide interactions in Rab function (6–8). The Rab6 protein is likely involved in intra-Golgi transport. A GTPase defective Rab6 greatly reduced transport of the proteins between cis/medial and late Golgi compartments in mammalian cell culture (9). More recently, Martinez et al. (8) found that overexpression of wild-type Rab6 and a GTPase-defective Rab6 redistributed a trans-Golgi protein to the ER membrane compartment.

Biochemical studies using specialized cells, however, have suggested a role for Rab6 in post-Golgi transport. Rab6 is associated with post-Golgi vesicles in Torpedo marmorata electrocytes (10), hypothalamic neuronal cells (11), and frog retinal cells (12). The studies with frog retinal cells suggested that the Rab6 protein is associated with rhodopsin-containing vesicles that exit from the trans-Golgi on their way to the rod outer segment.

The Drosophila photoreceptor provides an excellent experimental system to study Rab6 function in rhodopsin membrane trafficking, given the availability of mutations in rhodopsin and other genes that impede rhodopsin maturation. Many of these mutations result in age-dependent degeneration of photoreceptors, suggesting that correct rhodopsin trafficking is critical to maintenance of photoreceptor stability. Some human retinal diseases caused by rhodopsin mutations, may also be due to improper rhodopsin trafficking within the photoreceptor (13). In addition, an inherited form of choroideremia results from a defective Rab escort protein-1, establishing that defects in Rab protein function are involved in other human degenerative diseases (14).

We established an in vivo system to study the role of Rab6 in the trafficking of rhodopsin and other photoreceptor proteins. Our results suggest that Rab6 is required for anterograde rhodopsin transport through the ER-Golgi complex. Further, defects in Rab6 trafficking also trigger retinal degeneration, strengthening the tie between defects in the rhodopsin maturation pathway and photoreceptor degeneration.

**EXPERIMENTAL PROCEDURES**

Cloning of Drab6—Degenerate primers based on the conserved DTAGQ and NKXD sequence motifs found in all Rab proteins were used to RT-PCR amplify rab sequences from total Drosophila RNA. RNA was isolated following methods of Cathala et al. (15). RT-PCR reaction was performed as specified by the RT-PCR reaction kit manufacturer (Perkin-Elmer). The 170-base pair fragments recovered from these reactions were cloned and sequenced to identify the Drosophila rab6 sequence (Drab6). The 170-base pair fragment of Drab6 was then used to isolate the entire rab6 gene from a Drosophila genomic library. In situ hybridizations, carried out as described by Ashburner (16), placed the gene at 33C/D on the standard Drosophila salivary chromosome map.

**Site-directed Mutagenesis and Construction of Transgenic Flies**—Site-directed mutagenesis was used to create Drab6Q71L (AAC to ATC) and Drab6N125I (CAG to CTG). The coding sequence of the two mutants and Drab6Q71L were placed under the control of the ninaE promoter and inserted in a P-element transformation vector (17). Drosophila transgenic flies were made by standard means (18) using the null mutant ninaEΔ77 as the recipient strain. Four independent lines were obtained for Drab6Q71L and Drab6N125I and two independent lines were obtained...

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¹ The abbreviations used are: GDI, guanine nucleotide dissociation inhibitor; RT-PCR, reverse transcription-polymerase chain reaction; ERG, electroretinography; PDA, prolonged depolarizing afterpotential; ER, endoplasmic reticulum.
for Drab^Q71L. All lines for each construct showed similar levels of Rab6 expression and rhodopsin defects as described in this paper.

Generation of Rab6 Antibody—A polyclonal antibody to Drosophila Rab6 was generated using the GEX glutathione S-transferase system (19). To generate the antibody, a 249-base pair region coding for a C-terminal region of Rab6 (amino acids 129–298) was placed in the pGEX-3 vector. The fusion protein was collected on glutathione-agarose beads and then recovered from the beads by eluting in 8 M urea, 0.1 M EDTA, 100 mM β-mercaptoethanol, 0.1 M Tris, pH 8.0. The fusion protein was dialyzed overnight in 20 mM Tris, pH 8.0, and used to immunize mice.

Phenotypic Characterization—Proteins from fly heads were extracted in 60 mM Tris, pH 6.8, 25% glycerol, 2% SDS, 14.4 mM β-mercaptoethanol, and 0.1% bromphenol blue, separated by SDS-polyacrylamide gel electrophoresis (20) on 4–15%, 10%, or 12% gels, and transferred onto nitrocellulose membranes (Amersham Pharmacia Biotech) in 19 mM Tris, 150 mM glycine, 20% methanol. Proteins were detected using the polyclonal antibodies directed against Rh1 or Rh3 opsin,2 RdgB (21), Trp (22), and NinaC (23). Protein was detected using the ECL system (Amersham Pharmacia Biotech).

To analyze the transient expression of rhodopsin, we used a stock designated ha-ninaE-hsv tag 14. This stock contained an HSV (epitope identified in herpes simplex virus glycoprotein D)-tagged rhodopsin under the heat shock promoter (24). Flies heterozygous for the tagged rhodopsin and the respective Drab6 P-transgene were heat shocked for 1 h at 37 °C and placed at room temperature (22 °C) for the indicated time. Protein separation, transfer, and detection were performed as stated above, using a monoclonal antibody directed against the HSV tag (Novagen Corp.).

Electroretinography (ERG) recordings (as described in Larrivee et al. (25)) were performed on 2-day-old white eyed flies reared in a 12-h light/12-h dark cycle.

Electron microscopy was performed as described by Washburn and O’Toosa (26). All genotypes were white eyed and maintained in a 12-h light/12-h dark cycle. The control, Drab6^wt and Drab6^N125I flies were homozygous for ninaE^−, whereas the Drab6^Q71L flies were heterozygous for ninaE^−. 16 days old Drab6^wt flies heterozygous for rhodopsin were also sectioned and provided the same results (data not shown).

**RESULTS**

Drab6 Mutants Reduce Rhodopsin Levels—We used a PCR-based approach to initiate a study of Drosophila rab6 and identified the rab6 gene previously named Drab6 by Satoh et al. (27). We created two Drab6 mutations, the GTPase defective (Drab6^Q71L) and the guanine nucleotide binding defective (Drab6^N125I), by in vitro mutagenesis. These two mutants and the wild-type (Drab6^wt) coding sequences were placed under the control of the ninaE promoter to allow specific and high levels of expression only in the Drosophila R1–R6 class of photoreceptor cells (28). Protein blotting experiments using Drosophila Rab6 antibody confirmed that transgenic flies carrying these genes made large amounts of the Rab6 proteins (Fig. 1). The majority of the Rab6 protein in the transgenic flies possessed a higher apparent molecular mass than that seen in control wild-type flies, corresponding to a nonrenylated Rab6 protein (9). The modified form of Rab6 was also easily observed in these transgenic flies. We estimate that 32 times more lipid-modified Rab6 protein was detected in flies expressing the Drab6^wt constructs than in wild-type control flies. Similar high levels of modified Rab6 protein (37 times more protein in Drab6^N125I, and 57 times more protein in Drab6^Q71L) was observed in the other transgenic flies.

To look for generalized defects in photoreceptor function because of expression of these Rab6 proteins, we assayed the light response by ERG (Fig. 2). All strains show a robust response to light stimuli. A prolonged depolarizing afterpotential (PDA) is seen in the ERG, on exposure to blue light, when a substantial amount of rhodopsin is converted to an active metarhodopsin form (29). Control flies generate a complete PDA, and Drab6^wt and Drab6^N125I flies show a slight defect in the PDA maintenance. Drab6^Q71L flies completely lack a PDA. Given the importance of high rhodopsin levels in generating a PDA, these results suggested that Drab6^wt and Drab6^N125I have minor effects on rhodopsin expression, and Drab6^Q71L flies possess much lower levels of rhodopsin. Rhodopsin protein levels were examined by Western blot analysis to assess the effects of the Drab6 strains (Fig. 3). Rhodopsin levels are dramatically reduced in Drab6^Q71L (12% of wild type). There is a more modest reduction in the Drab6^wt and Drab6^N125I flies (76 and 74% of wild-type levels, respectively).

Drab6^Q71L Inhibits the Anterograde Transport of Rhodopsin—We analyzed the effects of the Drab6 strains in an experimental protocol designed to document defects in the rhodopsin maturation pathway (24, 30) (see also “Experimental Proce-
In these experiments, flies carried a rhodopsin gene tagged by an HSV epitope expressed from a heat shock promoter. Expression of this rhodopsin gene occurs only during a 37 °C heat shock, allowing the fate of rhodopsin synthesized during a restricted time window to be assessed. For the study here, we constructed strains carrying both the heat shock-controlled rhodopsin gene and each of the three Drab6 genes.

A strain containing the heat shock-controlled rhodopsin gene but no Drab6 construct served as the control in these experiments. In the absence of heat shock, no HSV-tagged rhodopsin could be detected in protein blotting experiments (Fig. 4A). Two h following the heat shock, the rhodopsin is detected as a 40-kDa species (open arrow) as well as several slightly higher molecular mass forms. 14.5 h after the pulse, rhodopsin is still present in the 40-kDa form but now is also detected in lower molecular mass bands (35–38 kDa). At 24 h after the heat shock, most of the rhodopsin is found in the 35-kDa form (filled arrow). This 35-kDa form has the same mobility as the major species of rhodopsin found in flies expressing the HSV-tagged rhodopsin from the ninaE promoter, hence we consider it the mature form. The strains containing the Drab6wt and Drab6N125I genes had the same profile as the control strain (Fig. 4, C and D). The Drab6Q71L flies, however, showed defects in rhodopsin maturation (Fig. 4B). Two h after heat shock, the majority of the rhodopsin was detected in the 40-kDa form, as expected from the analysis of the other strains. However, at 14.5, 24, and 48 h after heat shock, the 40-kDa rhodopsin remained as the predominant species. The data establish that the Drab6Q71L mutant is defective in processing the immature 40-kDa rhodopsin species into the mature 35-kDa form.

Drab6 Mutants Affect Opsin but Not Other Photoreceptor Proteins—In Drosophila six different opsins are expressed in subsets of the photoreceptor cells. To test the effects of the Drab6 constructs on a different rhodopsin, we misexpressed the Rh3 rhodopsin in R1–R6 photoreceptor cells (31). Western blot analysis of these strains (Fig. 5A) showed that Rh3 protein levels were reduced in all three Drab6 transgenic strains compared with controls. As with expression of the Rh1 (NinaE) protein, Rh3 levels were most reduced in the Drab6Q71L flies, with the other two lines showing a significant, but smaller, reduction of protein.

We examined the protein levels of two other photoreceptor proteins. In the absence of heat shock, no HSV-tagged rhodopsin could be detected in protein blotting experiments (Fig. 4A). Two h following the heat shock, the rhodopsin is detected as a 40-kDa species (open arrow) as well as several slightly higher molecular mass forms. 14.5 h after the pulse, rhodopsin is still present in the 40-kDa form but now is also detected in lower molecular mass bands (35–38 kDa). At 24 h after the heat shock, most of the rhodopsin is found in the 35-kDa form (filled arrow). This 35-kDa form has the same mobility as the major species of rhodopsin found in flies expressing the HSV-tagged rhodopsin from the ninaE promoter, hence we consider it the mature form. The strains containing the Drab6wt and Drab6N125I genes had the same profile as the control strain (Fig. 4, C and D). The Drab6Q71L flies, however, showed defects in rhodopsin maturation (Fig. 4B). Two h after heat shock, the majority of the rhodopsin was detected in the 40-kDa form, as expected from the analysis of the other strains. However, at 14.5, 24, and 48 h after heat shock, the 40-kDa rhodopsin remained as the predominant species. The data establish that the Drab6Q71L mutant is defective in processing the immature 40-kDa rhodopsin species into the mature 35-kDa form.

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We examined the protein levels of two other photoreceptor proteins.
Drab6 regulates Rhodopsin maturation in Drosophila photoreceptors. Drab6Q71L flies possess about 12% of the wild-type steady state levels of rhodopsin. Drab6N125I flies possess about 75% of the wild-type rhodopsin levels. The only deficit in the ERG traces can be attributed to the reduction in rhodopsin content, indicating that overexpression of the Rab6 proteins did not have a debilitating effect on the physiology of these photoreceptor cells.

Analysis of rhodopsin transport using a heat shock-regulated promoter demonstrated that in wild-type, Drab6Q71L, and Drab6N125I photoreceptors, rhodopsin matures to its final 40-kDa form within 24 h. In contrast, rhodopsin maturation is severely impaired in Drab6Q71L, showing little progression beyond the 40-kDa intermediate form. Previous work established that the 40-kDa rhodopsin is a high mannose intermediate found within the ER. The 40-kDa rhodopsin requires the ninaA encoded cyclophilin (35) and retinal addition (36, 37) to exit the ER. Our results show that Rab6Q71L blocks rhodopsin transport prior to its progression into the cis or medial Golgi compartment that contains the mannosidase which acts on the high mannose rhodopsin intermediate (38, 39). These results are consistent with a role of Rab6 in intra-Golgi transport. Although we have no data suggesting Rab6 in post-Golgi events as suggested by a study on frog retinal cells (12), our analysis does not rule out a second independent role of Rab6.

A recent study showed that transient expression of Rab11241l protein prevents rhodopsin maturation beyond the 40-kDa intermediate (40). Similar to the phenotype observed in the Drab6Q71L mutant. It is striking that dominant mutants of the first two Rab proteins studied in the Drosophila photoreceptor appear to affect similar stages of rhodopsin maturation. However, rhodopsin likely remains in a 40-kDa form as it trafficks from the ER to the cis or medial Golgi where modifications of the oligosaccharide side chain is thought to occur. Therefore, multiple Rab, including the Rab1 and Rab6 proteins, may be required in these steps. The expression of Rh3 rhodopsin was also markedly reduced in the Drab6Q71L flies, and smaller effects were seen in the two other Drab6 strains. On the other hand, none of the Drab6 strains affected the levels of other photoreceptor proteins tested. These results suggest that rhodopsin transport is more sensitive to defects in the Rab6-regulated pathway, with alternative maturation pathways available for other photoreceptor membrane proteins. An alternative explanation, that RdgB and Trp are transported via an alternative pathway cannot be excluded. Resolutions of these issues will likely require the identification and analysis of an in vivo loss of function Rab6 mutant.

**DISCUSSION**

**Rab6 Role in Rhodopsin Maturation—** A major objective of this study was to investigate the role of Rab6 in rhodopsin maturation. Protein blotting experiments and ERG analysis established that Drab6Q71L flies possessed about 12% of the wild-type steady state levels of rhodopsin. Drab6N125I flies possess about 75% of the wild-type rhodopsin levels. The only deficit in the ERG traces can be attributed to the reduction in rhodopsin content, indicating that overexpression of the Rab6 proteins did not have a debilitating effect on the physiology of these photoreceptor cells.
The Effect of Dominant Forms of Rab6—By analogy with point mutations of rab6 (9) and other rab genes (6, 41), the Gln to Leu change prevents GTP hydrolysis. Therefore the Rab6Q71L protein will always be bound to GTP.

**Drab6Q71L** is a potent inhibitor of rhodopsin protein transport, which is consistent with the behavior of this mutation in other Rab6 studies (8, 9). If GTP hydrolysis is required for vesicle fusion, as proposed for Rab3a (42), Drab6Q71L is expected to prevent the fusion of vesicles with their target membrane. Our results showing that the Drab6Q71L form inhibits rhodopsin transport is consistent with a role for GTP hydrolysis to promote anterograde transport of rhodopsin-bearing vesicles. Alternatively, Rab6 in its GTP form could be a positive regulator of the retrograde transport, as proposed by Martinez et al. (8, 9). According to this notion, Drab6Q71L could increase the flow of retrograde transport and indirectly disrupt the anterograde pathway, resulting in inhibition of protein transport. However, this model was originally proposed to rationalize results showing that Rab6wt has similar effects as Rab6Q72L that were not confirmed in our experiments.

We also documented an inhibition of Rh1 and Rh3 rhodopsin expression in the Drab6wt and Drab6N125I strains. However, the heat shock analysis indicates that Drab6wt and Drab6N125I have little or no inhibitory effects on the maturation of the 40- to the 35-kDa form of Rh1 rhodopsin. Thus, the mechanism of Drab6wt and Drab6N125I action is distinct from that of Drab6Q71L. The Drab6N125I and Drab6Q72L proteins might have an effect on later stages of rhodopsin maturation, but it is also possible that the reduction in rhodopsin is a consequence of secondary effects associated with the overexpression of these proteins. All Rab proteins require isoprenylation to be functional (43). When we overexpress Rab6 in photoreceptors, 25–35% of the protein is isoprenylated. The failure to completely modify the large amount of Rab6 found in these flies suggests that overexpression has overwhelmed the Rab geranylgeranyl transferase pathway responsible for the prenylation of all Rab proteins (46). Therefore, overexpression of Rab6 may also inhibit the modification, and therefore the activity, of other Rab proteins. Thus, the defects seen in photoreceptors overexpressing Rab6wt or Rab6N125I may not be directly attributable to the altered Rab6 activity.

It is surprising that Drab6wt and Drab6N125I have similar inhibitory effect on secretion as observed for the Gln to Leu mutations (6, 42). On the other hand, the Asn to Ile mutation in rab6 increased secretion rate (7). The lack of a mutant phenotype in our studies does not result from Rab6N125I protein instability since protein immunoblots show high levels of this protein. It appears that the Rab6N125I protein, perhaps because of lack of nucleotide binding, is unable to participate in the Rab6 cycle.

**Effects of Rab6 on Photoreceptor Degeneration—Overexpression of any form of Rab6 caused retinal degeneration, but the rate and severity of degeneration depended upon the form of Rab6. At young ages, Drab6Q71L photoreceptors already show structural differences that distinguish it from Drab6wt and Drab6N125I photoreceptors. The most striking difference is a much smaller volume occupied by the R1–R6 rhabdome. This phenotype is shared with mutant ninaE (33, 44), ninaA (35), ninaC (45, 46), as well as vitamin A deprived flies (47). All these flies possess reduced rhodopsin content, suggesting that the reduced size of the rhabdome in the Drab6Q71L mutant is likely the result of poor rhodopsin maturation.

The Drab6Q71L photoreceptors exhibit other ultrastructural defects, most notably an accumulation of disorganized membranes within the cytoplasm as well as “whorl” membranes thought to represent membrane recycling processes (34). Satoh et al. (40) documented a similar phenotype in the Drosophila rab1N124I mutant. Consistent results are also obtained in mammalian cell culture. Martinez et al. (8) documented that overexpression of the rab6Q72L mutant allows the mixing of ER and Golgi membrane compartments, and morphological changes of the ER/Golgi are noted in other studies using lovastatin to limit prenylation of Rab proteins (48). Thus, the abnormal membrane accumulation documented in Drab6Q71L photoreceptors may result from abnormal Golgi organization, and the defects in rhodopsin maturation may be a secondary consequence of this defect. On the other hand, our data are not compatible with a catastrophic defect in ER-Golgi transport in Drab6Q71L photoreceptors, as these photoreceptors retain normal physiological function, and other membrane proteins are detected at normal levels.

Dominant rhodopsin mutants cause age-dependent retinal degeneration as a result of defects in rhodopsin transport (30, 49). We initiated this study to examine the role of Rab6 in rhodopsin transport and to explore an in vivo experimental system to study the trafficking of rhodopsin. Our results establish the importance of Rab6-regulated trafficking mechanisms.
in both rhodopsin biogenesis and maintenance of photoreceptor morphology and function.

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