GPI biosynthesis is essential for rhodopsin sorting at the trans-Golgi network in Drosophila photoreceptors

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SUMMARY
Sorting of integral membrane proteins plays crucial roles in establishing and maintaining the polarized structures of epithelial cells and neurons. However, little is known about the sorting mechanisms of newly synthesized membrane proteins at the trans-Golgi network (TGN). To identify which genes are essential for these sorting mechanisms, we screened mutants in which the transport of Rhodopsin 1 (Rh1), an apical integral membrane protein in Drosophila photoreceptors, was affected. We found that deficiencies in glycosylphosphatidylinositol (GPI) synthesis and attachment processes cause loss of the apical transport of Rh1 from the TGN and mis-sorting to the endolysosomal system. Moreover, Na⁺K⁺-ATPase, a basolateral membrane protein, and Crumbs (Crb), a stalk membrane protein, were mistransported to the apical rhabdodermal microvilli in GPI-deficient photoreceptors. These results indicate that polarized sorting of integral membrane proteins at the TGN requires the synthesis and anchoring of GPI-anchored proteins. Little is known about the cellular biological consequences of GPI deficiency in animals in vivo. Our results provide new insights into the importance of GPI synthesis and aid the understanding of pathologies involving GPI deficiency.

KEY WORDS: GPI, Rhodopsin, Sorting, Drosophila

INTRODUCTION
The biosynthesis of glycosylphosphatidylinositol (GPI)-anchored proteins has been thoroughly investigated in cultured cells and yeasts for 20 years, resulting in the identification of a number of genes involved in GPI anchoring, with names originating from PIG, phosphatidylinositol glycan (Fujita and Kinoshita, 2010; Maeda and Kinoshita, 2011). However, little is known about the phenotypic consequences of dysfunction in the GPI pathway in vivo. Knockout of Pig-a (Piga – Mouse Genome Informatics), a component of GPI-GlcNAc transferase and the enzyme at the first step of GPI biosynthesis, causes embryonic lethality in mice (Nozaki et al., 1999). Somatic mutations in the PIG-A (PIGA – Human Gene Nomenclature Database) gene in hematopoietic stem cells are associated with paroxysmal nocturnal hemoglobinuria (Takeda et al., 1993). A hypomorphic promoter mutation in PIGM, a mannosyltransferase-encoding gene, causes venous thrombosis and seizures (Almeida et al., 2006). PIGY, which encodes α1,6-mannosyltransferase II, was identified as the gene mutated in hyperphosphatasia mental retardation (Krawitz et al., 2010). As expected, deficient GPI anchoring or remodeling causes lost or delayed transport or secretion of GPI-anchored proteins. Furthermore, recent studies in yeast and zebrafish show that the transport of non-GPI-anchored proteins is also affected by abolishment of GPI biosynthesis (Okamoto et al., 2006; Nakano et al., 2010).

Drosophila photoreceptors are well suited for studying polarized transport, because a single retinal cross section simultaneously shows three distinct plasma membrane domains of numerous photoreceptors (Fig. 1A–C). One is the photoreceptive membrane domain, known as the rhabdomere, which is formed at the center of the apical plasma membrane as a column of closely packed rhodopsin-rich photosensitive microvilli (Fig. 1B,C). The second is the peripheral apical domain surrounding the rhabdomere, termed the stalk membrane, where Crb and β-spectrin are enriched (Izaddoost et al., 2002; Pellikka et al., 2002). The third is the basolateral membrane, which is separated from apical membrane by adherens junctions, similar to the typical polarized epithelial cells. 

MATERIALS AND METHODS
Drosophila stocks and genetics
Flies were grown at 20-25°C in a 12-hour light/12-hour dark environment on standard cornmeal-glucose-agar-yeast food unless noted.

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otherwise. Carotenoid-deficient food was prepared from 1% agar, 10% dry yeast, 10% sucrose, 0.02% cholesterol, 0.5% propionate and 0.05% methyl 4-hydroxybenzoate. The fly stocks obtained from Bloomington (BL), the Kyoto Drosophila Genetic Resource Center (KY) or Harvard (HV) stock centers are referred to with their respective sources and stock numbers.

To visualize the genetic mosaic on the retina, 3xP3-RFP transgenes (Bischof et al., 2007), which express red fluorescent protein (RFP) in photoreceptors under the control of an artificial 3xP3 promoter, were recombined with proximal neorFT transgenes on the same chromosome arm as follows: M(3xP3-RFP.attP)ZH-2A (BL24480) and neoFRT19A; M(3xP3-RFP.attP)ZH-2A (BL24481) and neoFRT40A; M(3xP3-RFP.attP)ZH-584 (BL24484) and neoFRT42D; M(3xP3-RFP.attP)ZH-68E (BL24485) and neoFRT80B; and M(3xP3-RFP.attP)ZH-96E (BL24487) and neoFRT82B. The following test lines were used for live-image screening: y w P3RFP FRT19A; RhlArr2GFP eye-FLP/SM1, w; P3RFP FRT40A/SM1; RhlArr2GFP eye-FLP/TM6B, w; FRT42D P3RFP; RhlArr2GFP eye-FLP/TM6B, w; RhlArr2GFP eye-FLP/SM1; P3RFP FRT80B, w; RhlArr2GFP eye-FLP/SM1; and FRT82B P3RFP. Similar fly stocks without RhlArr2GFP transgene were used for the immunostaining of mosaic retina.

To generate deletion mutants of PGAP3 by imprecise excision, a P-element insertion on the 5’ of PGAP3 gene, P(Supor-P)[KG03595](BL3019), was outcrossed to w118 six times and subsequently excised using delta2-3 (BL3629). The deletion was analyzed by PCR using KOD-FX DNA polymerase and the primers 5’-TGGCAAACACAGAATGTCG-3’ and 5’-TCGTAATACCGCCGCTTGTA-3’.

GlcT-1 deletion mutants were generated using a standard induced FLP/FRT recombination method (Parks et al., 2004). Trans-heterozygous males of PBac(RB)p2597 (HVc2597) and FRT42D PBac(RB)2644 (KY114503) carrying hs-FLP (BL6876) were heat treated three times at 37°C for 1 hour during their larval stages. SM6a-balanced offspring were genotyped using PCR to select recombinants carrying both the proximal side of e2644 and the distal side of e2597 with the following primers: 5’-TATTTTTGCTGGGTTCCGAGT-3’ and 5’-TCCAAGCGCCGACTGAGATG-3’ for e2644, and 5’-CCTCGATATACAGCGGTATAACAC-3’ and 5’-GTGAAAGGTGCCGATGGTA-3’ for e2597.

Coding insertions of PIG-C PBac[WH]f05249, PIG-V PBac[WH]f05618, and PIG-V PBac[WH]f05618, G4AI P/Epox2/HVf05618 (BL17363), PIG-SP(GSV6)_f05249 (KY205476) and PGAP5 Mf/ET1p17572 (BL29250) were recombined by meiotic recombination with FRT80B, FRT42D, FRT19A, FRT82B and FRT40A, respectively. Mosaic retinas were visualized by mating with 3xP3RFP-FRT chromosomes, except PGAP5 Mf/ET1p17572, which has a 3xP3-GFP marker.

For the partial rescue experiment, the entire coding sequence of the PIG-C (PIGC – Human Gene Nomenclature Database) gene was cloned into pPTW to construct pPIGC and transgenic lines were generated by BestGene (Chino Hills, CA, USA).

**Live-image screening**
Each mutant line obtained from the Kyoto Drosophila Genetic Resource Center (DGRC) (supplementary material Table S1) was mated with the test lines. Late pupae of the siblings were attached to glass slides using double-sided sticky tape, and the pupal cases around the heads were removed. The pupae were chilled on ice, embedded in 0.2–0.5% agarose, and observed using an FV1000 confocal microscope equipped with LUMPlanFI water-immersion 40× objective (Olympus, Tokyo, Japan).

**Live imaging of fluorescent proteins expressed in photoreceptors**
For live-image screening, mutant flies obtained from the Kyoto DGRC were mated with the test lines carrying FRT, 3xP3-RFP, eye-FLP and RhlArr2GFP.
Construction of the antibody against *Drosophila* Rh1

Chicken affinity-purified anti-Rh1 antibody was raised against the Rh1 peptide GSVVDKVTDPDMHGIS (amino acids 21-36) (BioGate, Gifu, Japan). The antibody recognizes the band of Rh1 in western blots, previously identified by another anti-Rh1 monoclonal antibody, 4C5.

**Immunohistochemistry**

Fixation and staining were performed as described previously (Satoh and Ready, 2005). Primary antisera were as follows: mouse monoclonal anti-Rh1 [4C5; 1:20 supernatant; Developmental Studies Hybridoma Bank (DSHB), Iowa, IA, USA], rabbit anti-Rh1 (1:1000) (Satoh et al., 2005), chicken anti-Rh1 (1:1000; made in the present study), rabbit anti-GM130 (1:300; gift from Dr Ziker, Columbia University, NY, USA), mouse monoclonal anti-Na⁺/K⁺-ATPase alpha subunit (1:500 ascites; DSHB), rat monoclonal anti-DE-Cad (1:20 supernatant; DSHB), mouse monoclonal anti-Chp (24B10; 1:20 supernatant; DSHB), mouse monoclonal anti-Arm (1:20 supernatant; DSHB), rat anti-Crb (gift from Dr Tapass, University of Toronto, ON, Canada), rabbit anti-transient receptor potential (TRP; gift from Dr Montell, the Johns Hopkins University, MD, USA), rabbit anti-Rab7 (1:1000) and rat anti-Rbsn5 (1:1000; gift from Dr Nakamura, Riken, Kobe, Japan). The secondary antibodies were anti-mouse, anti-rabbit, anti-rat and anti-anti-chicken labeled with Alexa Fluor 488, 568, or 647 (1:300; Invitrogen, Carlsbad, CA, USA) with or Cy2 (1:300; GE Healthcare Life Sciences, Pittsburgh, PA, USA). Samples were examined using an FV1000 confocal microscope (60×1.42-NA lens) and images were recorded. To minimize bleed through, each signal in double- or triple-stained samples was imaged sequentially. Images were processed in accordance with the guidelines for proper digital image handling using Image J and/or Adobe Photoshop CS3.

**Filipin staining**

Filipin (1 mg; Sigma-Aldrich, St Louis, MO, USA) was dissolved in 400 µl DMSO (2.5 mg/ml) as a stock solution. Eyes were fixed and washed with 1× PBS and stained with 50 µg/ml filipin and Alexa Fluor 488-conjugated phalloidin (Invitrogen) for 2 hours and observed using an LSM710 confocal microscope (Carl Zeiss, Oberkochen, Germany).

**Blue light-induced chromophore supply (BLICS) method**

Newly eclosed flies fed carotenoid-deficient food were transferred to carotenoid-deficient food with crystalline all-trans retinal (Sigma-Aldrich) in the dark. After 1 or 2 days in the dark, the flies were irradiated with blue light (410 nm) using a CFP filter on a 75-W xenon lamp to isomerize the all-trans retinal to the 11-cis-form and initiate Rh1 maturation.

**Electron microscopy**

The conventional electron microscopic methods used are described by Satoh et al. (Satoh et al., 1997). Samples were observed on JEM1200 and JEM1400 electron microscopes (JEOL, Tokyo, Japan).

**Preparation of detergent-resistant fraction**

A detergent-resistant membrane was prepared using a rapid method as described previously (Adam et al., 2008). Preparation was carried out at 4°C in buffer-A (30 mM NaCl, 5 mM EDTA and 20 mM HEPES; pH 7.5) containing 1:200 Protease Inhibitor Mix III (Calbiochem). Total membranes were prepared from the pupal heads of Rip11 dominant-negative mutants (w; Rh1-Gal4/UA5-drIpl11-DN-GFP) or frozen heads of 0- to 1-day-old w1118 adults. The heads were homogenized in buffer-A using Biomasher II (Assist, Tokyo, Japan). The homogenates were centrifuged at 860 g for 3 minutes to remove the cuticle. The supernatants were centrifuged at 21,500 g for 30 minutes to collect the total membrane fraction. About 40 µl precipitated membranes was resuspended in 60 µl buffer-A, mixed with an equal volume of 2% Triton-X in buffer-A, and incubated on ice for 1 hour. The lysates were centrifuged at 21,500 g for 30 minutes to separate detergent-resistant and detergent-soluble fractions, then dissolved in sodium dodecyl sulfate sample buffer to a final volume of 100 µl. The fractions were then analyzed by immunoblotting 10 µl each for anti-TRP and 4C5 monoclonal anti-Rh1 antibodies.

**Immunoblotting**

Immunoblotting was performed as described previously (Satoh et al., 1997). The following antibodies were used: mouse monoclonal anti-TRP (1:3000; DSHB) and mouse monoclonal anti-Rh1 (4C5; 1:5000 concentrated supernatant; DSHB) as primary antibodies and HRP-conjugated anti-mouse antibody (1:20,000, Jackson Immunoresearch Laboratories) as a secondary antibody. Primary antibodies were incubated overnight at 4°C, and secondary antibody incubation and washing were performed using the SNAP-id Protein Detection System (Millipore, Billerica, MA, USA). Signals were visualized using enhanced chemiluminescence (ECL; GE Healthcare Life Sciences).

**RESULTS**

**Live-image screening of mutants exhibiting Rh1 transport defects in fly photoreceptors**

To identify which genes are essential for Rh1 transport, we performed retinal mosaic screening using the FLP/FRT method (Xu and Rubin, 1993) and two-color fluorescence imaging, similar to a method described recently (Gambis et al., 2011). In our screening, RFP was used as a wild-type cell marker and Arrestin2::GFP (Satoh et al., 2010) was used to visualize endogenous Rh1 localization. The corea neutralization technique was applied to observe the phenotype in vivo (supplementary material Fig. S1A,B) (Mollereau et al., 2000; Pichaun and Desplan, 2001). As Arrestin2::GFP was expressed in all R1-R6 peripheral photoreceptors in the mosaic retina, the localization and amounts of Rh1 could be compared between wild-type and mutant cells within the same optical section.

We screened 546 lines of lethal insertions of *P-element* or piggyBac transposons in the University of California, Los Angeles Undergraduate Research Consortium in Functional Genomics (UCLA URCFG) collection (Chen et al., 2005) using the two-color live-imaging method (supplementary material Table S1). For selected lines that showed some deficiency, the distributions of Rh1 and Na⁺/K⁺-ATPase were investigated by immunostaining to observe the phenotypes of transport and morphogenesis. Among the lines exhibiting severe Arrestin2::GFP reduction with only minor omatidial disorganization, two lines, KY111587 (Fig. 1D) and KY114591 (data not shown), had an insertion on the 5’ UTR or promoter region of a particular gene, *CG13089*. Immunostaining of these two mosaic retinas with anti-Rh1 revealed a dramatic reduction of Rh1 in the rhabdomeres (Fig. 1E; data not shown). This phenotype was rescued by the removal of the *P-element* insertion in both lines (supplementary material Fig. S1C,D), indicating that the Rh1 reduction is caused by the reduction of *CG13089* gene function. *CG13089* has 38% identity and 57% similarity to the human *PIG-U* (*PIGU* – Human Gene Nomenclature Database) gene, which encodes a subunit of GPI-transamidase (supplementary material Table S2). As Rh1 is an integral membrane protein and is not anchored by GPI, these results suggest that the formation of GPI-anchored proteins is also important for the trafficking of non-GPI-anchored proteins to the rhabdomeres.

**Roles of GPI synthesis and remodeling in Rh1 transport**

The *PIG-U* alleles identified in our screening are hypomorphic; no *PIG-U*-null mutant was available. To investigate further the role of GPI synthesis and anchoring in vivo, we searched for null mutants of other *PIG* genes in the genome of *Drosophila melanogaster* (supplementary material Table S2). There were four available insertions in the coding regions of *PIG* genes: *PIG-C* (Genbank accession number: GAA1EY069050), *PIG-D* (Genbank accession number: GAA1EY069050) and *PIG-S* (Genbank accession number: GAA1EY069050) (supplementary material Fig. S1E).
As all four null mutants are embryonic (i.e. PIG-S<sup>S154474</sup> or larval lethal (i.e. PIG-C<sup>Y05249</sup>, PIG-V<sup>Y05618</sup> and GAA<sup>YEn9065</sup>), we recombinated them with FRT chromosomes to make mosaic retinas. We first investigated Rh1 localization in PIG-null homozygous clones (Fig. 1F-I). The severe reductions of Rh1 in PIG-C<sup>Y05249</sup>, PIG-S<sup>S154474</sup> and GAA<sup>YEn9065</sup> mutant cells (asterisks) indicate that GPI synthesis and/or anchoring is essential for Rh1 synthesis or transport. PIG-V<sup>Y05618</sup> mutant photoreceptors exhibited less Rh1 reduction in the rhabdomeres than any other allele tested. The mutant clone sizes in PIG-V<sup>Y05618</sup> mutant retinas were larger than those of the other three null mutants: >13% of ommatidia in PIG-V<sup>Y05618</sup> mosaics were composed of only mutant cells compared with <3% in the other three mutants (supplementary material Fig. S1E). These results are concordant with the smaller Rh1 transport deficit observed in PIG-V<sup>Y05618</sup> mutants relative to that in the other three nulls. The loss of PIG-V activity in humans results in reduced anchoring of alkaline phosphatase (ALP) to the surface membrane and elevated ALP activity in blood serum (Krawitz et al., 2010). The recent explanation for this phenomenon states that GPI transamidase recognizes incomplete GPI-bearing mannose and cleaves a hydrophobic signal peptide, resulting in the secretion of soluble ALP (Murakami et al., 2012). Similarly, proteins having partial functioning without GPI anchoring might be released from the ER to the secretory pathway in fly PIG-V<sup>Y05618</sup> mutant photoreceptors, which could result in the milder phenotype of the PIG-V null mutant. A recent study on PIG-V shows a similar but milder Rh1 reduction in PIG-V<sup>A276F</sup>, which has an alanine-to-valine substitution at amino acid position 276V (Rosenbaum et al., 2012).

The structures of the lipid and glycan moieties on GPI anchors are remodeled during biosynthesis and after attachment to proteins (Fujita and Kinoshita, 2010). Lipid remodeling is required for the proper transport and raft association of GPI-anchored proteins (Maeda and Kinoshita, 2011). The proteins involved in these processes are called post-GPI attachment proteins (PGAPs) (supplementary material Fig. S1F). We searched the genomic sequences of D. melanogaster for PGAP genes and found a lethal insertion, PGAP<sup>5MB1732</sup>, containing a transposon insertion in exon 2 of PGAP5 (supplementary material Table S2). We recombinated PGAP5<sup>5MB1732</sup> onto the FRT40A chromosome and investigated Rh1 localization in mosaic clones. Unexpectedly, Rh1 localized normally in rhabdomeres in PGAP5-null photoreceptors (supplementary material Fig. S1G). To obtain a PGAP3-null mutant, we created deletion mutants of the PGAP3 gene using imprecise excision of a P-element insertion, PGAP3<sup>KG01356</sup> (supplementary material Fig. S1H). We obtained two PGAP3-null mutants, PGAP3<sup>AA81</sup> and PGAP3<sup>AF10</sup>, both were homozygous viable and exhibited normal Rh1 localization (supplementary material Fig. S1I; data not shown). These results indicate that GPI synthesis but not GPI remodeling is essential for Rh1 delivery to the rhabdomere.

**Chaoptin accumulation in the ER of GPI-deficient photoreceptors**

As GPI-anchored proteins are expected to be not synthesized normally or to be secreted rather than binding to the membrane in GPI-deficient cells, we investigated the synthesis and transport of Chaoptin (Chp), a major GPI-anchored protein in fly photoreceptors (Krantz and Zipursky, 1990). In wild-type cells, Chp localizes at the rhabdome. However, in GPI-deficient cells, most Chp colocalizes with the Rh1 chaperone NinaA (Colley et al., 1991) in the ER; only a limited amount of Chp was detected in the rhabdomeres (Fig. 2A). Although GPI-free Chp was expected to be secreted, no Chp secretion to the inter-rhabdomeric space (IRS) was observed in any PG1 mutant. These observations indicate that GPI anchoring is essential for the ER exit of Chp. However, Chp localized normally to the rhabdomeres of both PGAP3 and PGAP5 mutant photoreceptors (data not shown), suggesting that GPI remodeling is not essential for Chp synthesis or transport.

**Rhabdomeric membrane in PG1 mutant photoreceptors**

Electron microscopic observations of thin sections revealed that rhabdomeres in GPI-deficient photoreceptors were severely disrupted (Fig. 2B). In wild-type photoreceptors, rhabdomere microvilli were tightly packed and rhabdomeres had round cross-
sectional profiles. However, the rhabdomeres of GPI-deficient cells were small and irregularly shaped; the number of microvilli was greatly reduced, and the microvilli had different orientations and were not packed tightly (Fig. 2B, inset). Some microvilli in GPI-deficient photoreceptors had greater diameters and shorter lengths than the wild-type microvilli (Fig. 2B, black arrows in inset). Adherens junctions and the basolateral membrane (Fig. 2B, arrowheads and white arrows, respectively; supplementary material Fig. S2) as well as cytoplasmic organelles, the ER, Golgi bodies and mitochondria were normal (supplementary material Fig. S2). Therefore, the apical plasma membrane, especially the central rhabdomere domain, was specifically disrupted in GPI-deficient photoreceptors.

As shown in Fig. 2A, GPI-deficient rhabdomeres lacked Chp, which packs rhabdome microvilli through homotypic adhesion (Reinke et al., 1988). An allele of chp, chp², encodes a truncated form of Chp that lacks the GPI-attachment site (Krantz and Zipursky, 1990). This Chp N-terminal polypeptide in the chp² mutant was retained in the ER and was not transported to the rhabdomeres (Fig. 2D). The microvilli in chp² mutants are relatively short, have variable length and are separated from each other (Fig. 2C) as described previously (Krantz and Zipursky, 1990). However, the morphology of chp² mutant rhabdomeres was much less impaired than that of GPI-deficient photoreceptors. Most importantly, chp² mutants showed only a mild reduction of Rh1 accumulation in rhabdomeres (Fig. 2E). These results indicate that the deficiencies of Rh1 transport and rhabdomere structure in PIG mutants are not a consequence of the loss of Chp function.

**Synthesis, transport and degradation of Rh1 in PIG-deficient photoreceptors**

To identify the step of Rh1 synthesis or transport that is inhibited in PIG mutants, we clarified the dynamics of Rh1 transport using blue light-induced chromophore supply (BLICS) (Satoh et al., 1997; Satoh et al., 2005). Briefly, Rh1 comprises an apoprotein called opsin (also known as NinaE) and the chromophore 11-cis retinal. Without the chromophore, opsin accumulates in the ER. Blue-light illumination photoisomerizes retinal from all-trans to 11-cis, inducing the synchronous release of Rh1 from the ER into the secretory pathway.

Prior to BLICS, Rh1 apoprotein colocalized with ER markers (data not shown) in both wild-type and GPI-deficient photoreceptors (Fig. 3A,B, 0 minutes). Forty minutes after BLICS, Rh1 was concentrated in large dot-like structures, shown to be fly Golgi units, in both wild-type and GPI-deficient photoreceptors (Fig. 3A,B, 40 minutes). The colocalization of Rh1 with a Golgi marker confirmed the Golgi localization of Rh1 in GPI-deficient photoreceptors (Fig. 3C). These results indicate that Rh1 is normally synthesized and transported to Golgi units in GPI-deficient photoreceptors.

Sixty minutes after BLICS, Rh1 was not only concentrated in the Golgi units, but also localized at the base of the rhabdomeres, appearing as dot-like staining patterns in wild-type photoreceptors (Fig. 3A, 60 minutes). By contrast, in GPI-deficient photoreceptors, Rh1 was not localized at the base of the rhabdomeres but in irregular-shaped cytoplasmic organelles (Fig. 3B, 60 minutes) containing the endosomal markers Rab7 and Rab5 (Rbsn-5) (Tanaka and Nakamura, 2008) (Fig. 3E; data not shown). However, Rh1 and Rab7 or Rbsn5 did not colocalize in the wild-type cells (Fig. 3D; data not shown).

Rh1 transport was complete and Rh1 had strongly accumulated in the rhabdomeres in the wild-type cells by 180 minutes after BLICS (Fig. 3A, 180 minutes). However, in GPI-deficient photoreceptors, only a limited amount of Rh1 reached the rhabdomeres. Interestingly, Rh1 was not observed in the cytoplasm in GPI-deficient photoreceptors (Fig. 3B, 180 minutes). These results indicate that most Rh1 is degraded within 180 minutes after Golgi arrival in GPI-deficient photoreceptors. The colocalization of Rh1 with the endosomal markers Rbsn5 and Rab7 60 minutes after BLICS suggests that Rh1 is degraded by the endolysosomal
system (Fig. 3E; data not shown). To investigate this possibility, we introduced an lt1 homozygous viable mutation into PIG mosaic flies to inhibit lysosomal degradation. The lt gene encodes a homolog of yeast Vps41p, which is a component of the HOPS (homotypic vacuole fusion and protein sorting) complex. The degradation of endosomal cargo is inhibited in an lt1 homozygous background (Chinchore et al., 2009). We observed more Rh1-positive staining in the cytoplasmic organelles in PI C-lt1 double-mutant photoreceptors than in lt1 or PIG-C single-mutant cells (Fig. 3F versus Fig. 1F). Therefore, Rh1 is degraded by the endolysosomal system in PIG null-mutant photoreceptors.

**Epistatic analysis between GPI synthesis and the Rab11-Rip11-MyoV complex**

We previously showed that the Rab11-Rip11-MyoV complex is essential for post-Golgi vesicle transport and that a deficiency in any component of the complex induces the accumulation of Rh1-loaded post-Golgi vesicles in the cytoplasm (Satoh et al., 2005; Li et al., 2007). To investigate the epistatic interaction between the Rab11-Rip11-MyoV complex and GPI synthesis for Rh1 transport, we observed Rh1 localization in the PIG-V mutant mosaic retina expressing Rip11 dominant-negative protein. Rip11-PIG-V double-mutant photoreceptors did not accumulate Rh1 in the cytoplasm and the small amount of Rh1 localized in the rhabdomeres (Fig. 3G). This phenotype was indistinguishable from that of cells with only a PIG-V mutation (Fig. 1G). This result indicates that GPI synthesis is epistatic to the Rab11-Rip11-MyoV complex. Kinetic and epistatic analyses of Rh1 transport in GPI-deficient cells revealed that GPI synthesis is necessary for processes after Golgi entry and before/upon post-Golgi vesicle formation during Rh1 biosynthetic trafficking.

**Mislocalization of Na°K°-ATPase and Crb to the rhabdomeres in GPI-deficient photoreceptors**

In addition to their Rh1 transport deficiency, PIG mutants show another remarkable transport phenotype. Unlike other mutants identified in our screening (supplementary material Table S1), PIG mutants show rhabdomeric misrouting of a basolateral membrane protein, Na°K°-ATPase, and a stalk membrane protein, Crb. In wild-type 90% pupal development (pd) and older R1-R6 photoreceptors, Na°K°-ATPase localized only on the basolateral membrane. In GPI-deficient cells, significant amounts of Na°K°-ATPase were detected in the rhabdomere, whereas less Na°K°-ATPase was present on the basolateral membrane (Fig. 4A; supplementary material Fig. S3A-D). Crb specifically localized to the stalk membrane in wild-type photoreceptors; however, in GPI-deficient cells, Crb also localized to the rhabdomeres (Fig. 4B; data not shown). These results indicate that GPI deficiency induces the mislocalization of basolateral and stalk membrane proteins to the rhabdomere membrane. However, this mislocalization was not caused by defects in adherens junction formation, because adherens junctions formed normally (Fig. 2B, arrowheads) and a component of adherens junctions, DE-cadherin (Shotgun – FlyBase), exhibited normal localization in GPI-deficient ommatidia (Fig. 4C). Moreover, although continuous photoreceptor adherens junctions are not formed in a Crb mutant, crb11A22 (Pellikka et al., 2002), Rh1 and Na°K°-ATPase localized normally on the rhabdomeres and basolateral membrane, respectively (Fig. 4D; supplementary material Movies 1, 2). These results indicate that the defects of Rh1 transport and Na°K°-ATPase mislocalization in rhabdomeres in PIG mutants are not caused by disruption of the continuity of the adherens junctions.

**Exclusion mechanism of rhabdomere-directed transport vesicles**

The hypothesis that a lack of exclusion from post-Golgi vesicle precursors at the TGN in PIG mutants causes the mislocalization of Na°K°-ATPase is supported by the phenotype of the mutants of AP1γ and AP47, which are components of the AP1 complex...
Both results strongly support our hypothesis that microvilli showed better shapes (supplementary material Fig. S4C). Observations showed that the rhabdomeres were small but their retinas (supplementary material Fig. S4A,B). Electron microscopic observations revealed that rhabdomere structure profiles were much better at 58% and 73% pd than at the late-pupal stage (i.e. 80-100% pd) and that TRP accumulated normally in the rhabdomeres (Fig. 4E,F). Thus, not all rhabdomeric proteins require GPI synthesis to be delivered to the rhabdomere.

**Rh1 transport defects and Na\(^{+}\)K\(^{-}\)-ATPase mislocalization in earlier developmental stages and partially rescued flies**

A recent paper reports a similar Rh1 reduction in PIG-V hypomorphic mutant photoreceptors and explains that it is a secondary defect caused by the disruption of rhabdomeres by Chp deficiency (Rosenbaum et al., 2012). However, in our studies, the near-normal localizations of TRP and Syx1A in the rhabdomeres of null mutants and the BLICS analysis provide evidence that the Rh1 transport defect is not caused by the secondary defect. To rule out the possibility that the secondary defect causes Rh1 and Na\(^{+}\)K\(^{-}\)-ATPase transport defects, we performed two kinds of observations. First, we examined the earlier stages of eye development. Before 73% pd, Rh1 transport was already clearly defective and much Na\(^{+}\)K\(^{-}\)-ATPase had mislocalization in the rhabdomeres (Fig. 5B). The mislocalization of Na\(^{+}\)K\(^{-}\)-ATPase was clear even at 55% pd (Fig. 5A); however, we could not investigate Rh1 transport deficiency at this time point, because Rh1 expression starts at around 70% pd. Electron microscope observations revealed that rhabdomere structure profiles were much better at 58% and 73% pd than at the late-pupal stage (i.e. 80-100% pd) and that TRP accumulated normally in the rhabdomeres at both time points (Fig. 5A,C). Second, we examined late-pupal retinas (>80% pd) of partially rescued PIG-C mosaic retinas that expressed the PIG-C gene via eyeless-Gal4. Because eyeless-Gal4 induces PIG-C gene expression at an early stage of eye development but stops this induction before the mid-pupal stage, we can analyze the effect of PIG-C deficiency while avoiding its effect in early eye development. We still observed the PIG phenotype (i.e. Rh1 transport defect, Na\(^{+}\)K\(^{-}\)-ATPase mislocalization and Chp accumulation in the ER) on these partially rescued PIG-C mosaic retinas (supplementary material Fig. S4A,B). Electron microscopic observations showed that the rhabdomeres were small but their microvilli showed better shapes (supplementary material Fig. S4C). Both results strongly support our hypothesis that PIG genes are essential for Rh1 transport to and Na\(^{+}\)K\(^{-}\)-ATPase exclusion from the rhabdomeres.

**Sorting of Rh1 in the TGN and lipid raft model**

In polarized epithelial cells, most GPI-anchored proteins are transported to the apical membrane and associate with detergent-resistant membrane (DRM). Lipid rafts, which are normally isolated as DRM, are believed to play an important role in protein sorting at the TGN or the recycling endosomes of polarized epithelial cells (Rodriguez-Boulan et al., 2005). The lipid raft hypothesis states that lipid rafts, which are microdomains rich in glycosphingolipids and cholesterol, concentrate some fractions of apically destined proteins by their affinity (van Meer and Simons, 1998). Therefore, we investigated the lipid raft deficiency in PIG mutants and the association between DRM and Rh1. The rhabdomere is a cholesterol-rich membrane domain (Fig. 6A) (Sanxaridis et al., 2007). In GPI-deficient photoreceptors, cholesterol was less concentrated in the rhabdomeres (Fig. 6A) and diffused more in the basolateral membrane. This result suggests that raft formation in photoreceptors is affected in PIG mutants. However, the reduced filipin staining in the rhabdomeres of PIG mutants could be the result of the less-condensed rhabdomere membrane. At least part of rhabdomeric membrane can be isolated...
as DRM in fly photoreceptors, and a rabdohemeric protein, TRP, associates with DRM in a light-dependent manner whereas mature Rh1 does not (Sanxaridis et al., 2007). We first confirmed that TRP associates with DRM isolated from light-exposed wild-type heads and that Rh1 does not (Fig. 6B). Next, we tested whether newly synthesized Rh1 can be temporally associated with DRM in the post-Golgi vesicles. To obtain DRM from the post-Golgi vesicles, we isolated DRM from Rip11 mutant whole eyes (data not shown) and retinas expressing Rip11 dominant-negative protein, which accumulate post-Golgi vesicles carrying Rh1 in the cytoplasm. The results show that Rh1 did not associate with DRM in these mutants despite the strong association between TRP and DRM (Fig. 6B).

Next, we investigated whether the sorting phenotype can be observed in a mutant lacking glycosphingolipids, an essential raft component. *brainiac* (*brn*) encodes a UDP-N-acetylgalactosamine, βMan β1,3-N-acetylgalactosaminyltransferase (β3GlcNAc-transferase), and *egghead* (*egh*) encodes a GDP-mannose, βGlc β1,4-mannosyltransferase, with putative functions in sequential steps in the biosynthesis of the core structure of arthro-series glycosphingolipids (GlcNacβ1–3Manβ1–4Glcβ1-Cer). It is reported that *brn*/*egh* double mutants lack glycosphingolipids and accumulate monoglucosylated ceramide (Wandall et al., 2005). Rh1 and Na’K’-ATPase localized normally in the rhabdomeres and basolateral membrane, respectively, in *brn*/*egh* double-mutant photoreceptors (Fig. 6C). We also investigated the deletion mutants of the sole fly ceramide glucosyltransferase, GlcT-1, which is the first step of glycosphingolipid synthesis; the product itself was recently shown to play an essential role in apical sorting in nematodes (Zhang et al., 2011). The homozygous GlcT-1 mutant photoreceptors in the mosaic retina exhibited normal morphology and Rh1 and Na’K’-ATPase localization (Fig. 6D,E), indicating that the sorting defects observed in the PIG mutants are not due to the possible glucosyl ceramide reduction. In addition, Rh1 expression in cholesterol-deficient flies is not drastically reduced (Sanxaridis et al., 2007). These results collectively indicate Rh1 and Na’K’-ATPase sorting at the TGN in fly photoreceptors is not strongly affected by lipid raft deficiency.

**DISCUSSION**

In this study, we screened 546 lethal lines for potential defects in Rh1 by examining the localization of Arr2::GFP in FLP/FRT-mediated mosaic retinas using two-color fluorescence imaging. We found a mutation in the *Drosophila* homolog of human PIG-U, which encodes a subunit of GPI transamidase. Mutations in other genes of the GPI synthesis pathway but not in the GPI modification pathway gave rise to the same phenotype. Furthermore, the GPI-linked protein, Chp accumulates in the ER whereas the stalk membrane Crumbs protein and basolaterally localized Na’K’-ATPase were mis-sorted to the rhabdomere. We demonstrated that Rh1 is degraded before entering the post-Golgi vesicles but that Crb and Na’K’-ATPase are misrouted into vesicles destined for the rhabdomere in PIG mutant cells.

There are two reports concerning GPI requirements for the transport of transmembrane proteins. In zebrafish, GPI transamidase is essential for the surface expression of voltage-gated sodium channels (Nakano et al., 2010). In yeast, GPI synthesis is required for the surface expression of Tat2p tryptophan permease, which is associated with DRM in wild-type cells. In GPI-deficient yeast, Tat2p and Fur4p fail to associate with DRM and are retained in the ER (Okamoto et al., 2006). Although DRM forms in the ER in yeast, in mammalian cells, it is likely that DRM formation occurs only after Golgi entry (Rivier et al., 2010). The reason for this is thought to be that GPI lipid remodeling occurs in different places: the ER in yeast and the Golgi body in mammalian cells (Rivier et al., 2010). In mammalian cells, lipid rafts are postulated to concentrate some fractions of apically destined proteins owing to their affinity for the TGN (van Meer and Simons, 1988) or recycling endosomes (Rodriguez-Boulan et al., 2005).

Along with the raft model, there are two possible explanations for the sorting phenotype of PIG mutant fly photoreceptors: (1) the polarized sorting of Rh1 depends on its affinity for the raft/DRM and the raft/DRM is deficient in PIG mutants; (2) unidentified GPI-anchored protein(s) play crucial roles in the polarized sorting of Rh1 and Na’K’-ATPase, and the raft/DRM provides a platform for the GPI-anchored protein-MEGAPasin. The first model predicts raft/DRM deficiency in PIG mutants, Rh1 association with lipid rafts and a stronger phenotype caused by mutations in the genes involved in
GPI is essential for Rh1 sorting

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Supplementary material
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Fig. 7. Model of protein sorting at the TGN. In the wild-type TGN, one or more GPI-anchored protein localizes to the neck and/or inside budding post-Golgi vesicles destined for the rhabdomeres and recruits rhabdomere proteins but excludes others. By contrast, GPI-deficient TGNs do not contain GPI-anchored proteins and do not perform protein sorting. Consequently, most Rh1 is sent to the endocytic pathway and degraded by lysosomes whereas small amounts of Rh1, Na⁺K⁺-ATPase and Crb are loaded into post-Golgi vesicles for delivery to the rhabdomeres.

References


