**INTRODUCTION**

The biosynthesis of glycosylphosphatidylinositol (GPI)-anchored proteins has been thoroughly investigated in cultured cells and yeast for 20 years, resulting in the identification of a number of genes involving 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 (Takeda et al., 1993). An hypomorphic promoter mutation in PIGGM, 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 GPI deficiency (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 rhabdome, 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 rhabdome, 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, similarly to the typical polarized epithelial cells. Na⁺K⁺-ATPase specifically localizes on the basolateral membrane (Yasuhara et al., 2000). In addition to these three membrane domains, fly photoreceptors possess a fourth domain, the axon and synapses, which extend below the retina to the brain (not shown).

*Rhodopsin* 1 (Rh1), the rhodopsin expressed in R1–R6 cells, is the most accessible protein for investigating apical polarized transport in fly photoreceptors. This is because it is massively synthesized and transported in the late pupal stage (Kumar and Ready, 1995) and its exit from the endoplasmic reticulum (ER) can be triggered by illumination with blue light (Ozaki et al., 1993). We and other groups have shown that Rab1 is involved in transport from the ER to the Golgi (Satoh et al., 1997), Rab6 works in the early transport pathway (Shetty et al., 1998), the Rab11-Rip11-MyoV (Didum – FlyBase) complex is essential for the post-Golgi transport of Rh1 (Satoh et al., 2005; Li et al., 2007), and the exocyst complex is essential for the tethering of post-Golgi vesicles to the base of the rhabdome (Beronja et al., 2005). To elucidate further the molecular mechanism of Rh1 transport, we screened for mutants deficient in Rh1 transport and found that PIG genes are essential for Rh1 transport. Our analysis of Rh1 transport in GPI-deficient cells revealed that GPI anchoring is essential for the polarized sorting of integral membrane proteins at the trans-Golgi network (TGN).

**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...
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 neoFRT transgenes on the same chromosome arm as follows: M[3xP3-RFP.attP]ZH-24 (BL24480) and neoFRT19A; M[3xP3-RFP.attP]ZH-22A (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; Rh1Arr2GFP eye-FLP/SM1, w; P3RFP FRT40A/SM1; Rh1Arr2GFP eye-FLP/TM6B, w; FRT42D P3RFP; Rh1Arr2GFP eye-FLP/TM6B, w; Rh1Arr2GFP eye-FLP/SM1; P3RFP FRT80B, w; Rh1Arr2GFP eye-FLP/SM1; and FRT82B P3RFP. Similar fly stocks without Rh1Arr2GFP 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' side of PGAP3 was cloned into the 3xP3-RFP-FRT chromosomes, except for e2644 and the distal side of e2597 with the following primers: 5'-TATTTTGTCTGGTCTCAGGT-3' and 5'-TCCAACGGCGGACTGA-GATG-3' for e2644, and 5'-CCTCCGATACAGCCGATAAAC-3' and 5'-GTAGAAGGCGGGCCAGTTGA-3' for e2597.

Coding insertions of PIG-C pbAc[WHf05249] (HVf05249), PIG-V pbAc[WHf05618] (HVf05618), G4A1 P[EPgy2]EY06903 (BL17363), PIG-S P[GSy6]f05249 (KY205476) and PGAP5 M[ET1]MB1757 (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 MB1757, which has a 3xP3-GFP marker.

For the partial rescue experiment, the entire coding sequence of the PIG-C (PIG – Human Gene Nomenclature Database) gene was cloned into pPTW to construct pPTW(US-PIG-C) 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 Rh1Arr2GFP.
Construction of the antibody against Drosophila Rh1
Chicken affinity-purified anti-Rh1 antibody was raised against the Rh1 peptide GSVVDKVTMPMHLLS (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; Abcam, Cambridge, UK), rabbit anti-NinaA (1:300; gift from Dr Zuber, 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-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-Rhsn5 (1:1000; gift from Dr Nakamura, Riken, Kobe, Japan). The secondary antibodies were anti-mouse, anti-rabbit, anti-rat and 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/ UAS-drRip11-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 comea neutralization technique was applied to observe the phenotype in vivo (supplementary material Fig. S1A,B) (Mollerau et al., 2000; Pichaud 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 omittational 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; data not shown). This phenotype was rescued by the removal of the P-element insertion in both lines (supplementary material Fig. S1C,D; data not shown). This phenotype was rescued by the removal of the P-element insertion in both lines (supplementary material Fig. S1C,D; data not shown). This phenotype was rescued by the removal of the P-element insertion in both lines (supplementary material Fig. S1C,D; data not shown).

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-εG055249, PIG-γ055618, GAA1pY0965050 and PIG-śL0474 (supplementary material Fig. S1E).

DEVELOPMENT
As all four null mutants are embryonic (i.e. PIG-S^L04474) or larval lethal (i.e. PIG-C^65249, PIG-Y^65618 and GAA^ET09605), we recombined 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^65249, PIG-S^L04474 and GAA^ET09605 mutant cells (asterisks) indicate that GPI synthesis and/or anchoring is essential for Rh1 synthesis or transport. PIG-Y^65618-null mutant photoreceptors exhibited less Rh1 reduction in the rhabdomeres than any other allele tested. The mutant clone sizes in PIG-Y^65618-null mutant retinas were larger than those of the other three null mutants: >13% of ommatidia in PIG-V^f05618 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). Similarly, proteins having partial functioning without GPI anchoring might be released from the ER to the secretory pathway in fly PIG-V^null photoreceptors, which could result in the milder phenotype of the PIG-V null mutant. A recent study on PIG-V^null suggests that 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 rhabdomere. 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 PIG mutant. These observations indicate that GPI anchoring is essential for the ER exit of Chp. However, Chp localized normally to the rhabdomeres of both PIGAP3 and PIGAP5 mutant photoreceptors (data not shown), suggesting that GPI remodeling is not essential for Chp synthesis or transport.

**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 rhabdomere. 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 PIG mutant. These observations indicate that GPI anchoring is essential for the ER exit of Chp. However, Chp localized normally to the rhabdomeres of both PIGAP3 and PIGAP5 mutant photoreceptors (data not shown), suggesting that GPI remodeling is not essential for Chp synthesis or transport.

**Rhabdomeic membrane in PIG 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-

---

**Fig. 2. Essential roles of PIG genes in Chp transport and rhabdomere formation.** (A) PIG-C^65249 mosaic Drosophila eye immunostained by anti-Chp (green) and anti-NinaA antibodies (magenta). Asterisks show PIG-C-null photoreceptors. (B) PIG-V^f05618 mosaic ommatidium by electron microscopy. R1, R3 and R7 photoreceptors are wild type; R2, R4, R5 and R6 photoreceptors are PIG-V-null mutants. Adherens junctions (arrowheads) and basolateral membrane (white arrows) are indicated. Inset: High-magnification image of an R6 rhabdomere; black arrows show cross sections of the microvilli. (C) chp^2 mutant ommatidium by electron microscopy. (D) Chp immunostaining of chp^2 mutant ommatidium. (E) Rh1 immunostaining of chp^2 mutant ommatidium. Scale bars: 2 μm.
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 rhabdomere 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) (Sato 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 Rbsn5 (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

**Fig. 3. Kinetics of Rh1 transport.** (A, B) Immunostaining of Rh1 before and after BLICS in *Drosophila* wild-type (A) and PIG-C-mutant ommatidia (B). (C) Immunostaining of Rh1 (green) and Golgi marker, GM130 (magenta) 40 minutes after BLICS in PIG-C-mutant ommatidia. Asterisks show PIG-C-null photoreceptors. Arrows indicate Golgi units. (D, E) Immunostaining of Rh1 (green) and the endolysosome marker Rab7 (magenta) at 60 minutes after BLICS in wild-type (D) and PIG-C-mutant (E) ommatidia. Arrows show colocalization. (F) Projection image from five slices at 0.5-μm intervals of PIG-C-null mosaic eye with llt homozygous background. Asterisks show PIG-C-null llt double-mutant photoreceptors. (G) PIG-V-null mosaic eye expressing Rpl11 dominant-negative proteins by Rh1Gal4 driver. Asterisks show PIG-V-null photoreceptors. Scale bars: 2 μm.
Moreover, although continuous photoreceptor adherens junctions normally localize in PIG-deficient ommatidia (Fig. 4C). This 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, DE-cadherin (Shotgun – FlyBase), exhibited normal 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.

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 mislocalization 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 membranes 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.

Therefore, the defects in adherens junction formation do not explain the Rh1 transport deficiency or Na⁺K⁺-ATPase mislocalization in PIG mutants. Taken together, the mislocalizations of Na⁺K⁺-ATPase and Crb and deficiency in Rh1 transport indicate that the sorting machinery at the TGN concentrates Rh1 into post-Golgi vesicles destined for the rhabdomeres while excluding Na⁺K⁺-ATPase and Crb, and that this process requires GPI synthesis.

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 AP-1 complex...
involved in the recruitment of basolateral proteins in the TGN (Fölsch et al., 1999; Gonzalez and Rodriguez-Boulan, 2009; Benhra et al., 2011). In AP1γ454e6 and AP4γ5515243 mutants, Na’K’-ATPase was mistransported to the apical stalk membrane but not to the rhabdomeres (supplementary material Fig. S3E; data not shown). This mis-sorting phenotype of AP-1 mutants indicates that overloaded Na’K’-ATPase in the TGN can be misloaded into vesicles destined for the stalk membrane when it fails to be recruited into basolateral transport vesicles. Moreover, the fact that Na’K’-ATPase is still excluded from the rhabdomere even in the AP1 mutants suggests that rhabdomere-directed transport vesicles have a strict exclusion mechanism that excludes proteins with other destinations and that GPI-deficient cells lack this essential exclusion mechanism.

Next, we investigated the localization of other rhabdomeric transmembrane proteins including TRP and syntaxin 1A (Syx1A). In contrast to Rh1, the localizations of TRP and Syx1A were not substantially reduced in PIG mutants; near normal levels of TRP and Syx1A localized to 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 mislocalized 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, 1988). 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 rhabdomeric 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 whereas mature Rh1 does not (Fig. 6B). Next, we tested whether newly synthesized Rh1 can be temporarily 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. 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 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(s). 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 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.

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.

**Fig. 6.** Lipid raft-independent sorting of Rh1 at the TGN. (A) Filipin staining in Drosophila mosaic retina containing both wild-type and PIG-C-null mutant photoreceptors. Asterisks show PIG-C mutant cells. Scale bar: 5 μm. (B) Immunoblot analysis of the associations of TRP and Rh1 with DRM in wild-type and dominant-negative Rip11-expressing retina. Membranes of homogenates from illuminated wild-type heads (lanes 1 and 3) and illuminated Rip11DN/Rh1Gal4 heads (lanes 2 and 4) were separated into DRMs and detergent-soluble membranes (DSMs). (C) Immunostaining of Na’K’-ATPase (green) and Rh1 (magenta) in brn9PP4/brn9PP4 mosaic ommatidia. Scale bar: 2 μm. (D) Formation of GlcT-1 deletion mutants. Two transposon insertion lines, GlcT-1e2644 and GlcT-1e2597, containing the FRT sequence were used to make GlcT-1 deletion mutants using the FLP/FRT method. GlcT-1Δ8 includes a part of synaptobrevin in addition to GlcT-1. (E) Immunostaining of a cross section of a GlucT-1Δ8 ommatidium. Rh1 (magenta) and Na’K’-ATPase (green). Scale bar: 2 μm.
GPI is essential for Rh1 sorting

**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. RAFT formation. By contrast, the second model predicts that GPI deficiency produces a stronger phenotype than that caused by raft deficiency.

Our analysis of lipid raft deficiency (Fig. 6) does not support the first model in which the loss of polarized sorting of Rh1/Na+K+-ATPase in PIG mutants is a consequence of raft deficiency; instead, our results support the second model in which unidentified GPI-anchored protein(s) concentrate Rh1 and exclude Na+K+-ATPase and Crb from post-Golgi vesicles destined for the rhabdomeres. Thus, loss of the GPI-anchored sorting protein(s) might cause most Rh1 to be directed into the endocytic pathway and degraded by lysosomes while simultaneously allowing Na+K+-ATPase and Crb to be loaded into post-Golgi vesicles destined for the rhabdomeres (Fig. 7). Chp is the only GPI-anchored protein known to be expressed in fly photoreceptors (Stepp et al., 1988). However, chp mutant embryos do not exhibit any mislocalization phenotype of Rh1 or Na+K+-ATPase (Fig. 2E; supplementary material Fig. S3F). Identifying the GPI-anchored protein(s) responsible for the sorting in the TGN is an important step for understanding this mechanism of polarized transport.

The biosynthetic pathway of GPI-anchored proteins has been well elucidated, but little was known to date about the phenotypic consequences of the loss of GPI synthesis in vivo. The present study demonstrates that GPI synthesis is essential for the sorting of non-GPI-anchored transmembrane proteins, including Rh1 and Na+K+-ATPase, without obvious defects in adherens junctions. Human PIGM or PIGV deficiency causes seizures (Almeida et al., 2006) or mental retardation (Krawitz et al., 2010). These neurological disorders might be also caused by the mis-sorting of some transmembrane proteins in addition to the defects in the formation of GPI-anchoring proteins. Our findings aid the understanding of the pathology of diseases involving deficient GPI-anchoring protein synthesis.

**Acknowledgements**

We thank Drs A. Nakamura, U. Tepass, C. Montell and C. Zuber, who kindly provided fly stocks and reagents. We also thank the Bloomington Stock Center and the Drosophila Genetic Resource Center of the Kyoto Institute of Technology for fly stocks.


