The Gos28 SNARE Protein Mediates Intra-Golgi Transport of Rhodopsin and Is Required for Photoreceptor Survival*

Erica E. Rosenbaum, Eva Vasiljevic, Spencer C. Cleland, Carlos Flores, and Nansi Jo Colley

From the Department of Ophthalmology and Visual Sciences, Department of Genetics and The McPherson Eye Research Institute, University of Wisconsin, Madison, Wisconsin 53792

SNARE proteins play indispensable roles in membrane fusion events in many cellular processes, including synaptic transmission and protein trafficking. Here, we characterize the Golgi SNARE, Gos28, and its role in rhodopsin (Rh1) transport through Drosophila photoreceptors. Mutations in gos28 lead to defective Rh1 trafficking and retinal degeneration. We have pinpointed a role for Gos28 in the intra-Golgi transport of Rh1, downstream from α-mannosidase-II in the medial-Golgi. We have confirmed the necessity of key residues in Gos28’s SNARE motif and demonstrate that its transmembrane domain is not required for vesicle fusion, consistent with Gos28 functioning as a t-SNARE for Rh1 transport. Finally, we show that human Gos28 rescues both the Rh1 trafficking defects and retinal degeneration in Drosophila gos28 mutants, demonstrating the functional conservation of these proteins. Our results identify Gos28 as an essential SNARE protein in Drosophila photoreceptors and provide mechanistic insights into the role of SNAREs in neurodegenerative disease.

SNARE (soluble N-ethylmaleimide-sensitive factor-attachment protein receptor) proteins constitute a universal machinery required for virtually all membrane fusion events in eukaryotic cells and are thus required for diverse cellular processes, including cell migration, hormone signaling, enzyme release, synaptic transmission, and the trafficking of proteins and lipids through intracellular compartments of the secretory pathway (1–3). Because of their central roles in fundamental cellular processes, SNARE proteins have been linked to a wide variety of human diseases. Of particular significance, SNARE proteins constitute an extensive superfamily containing at least 39 members in humans and 24 members in Drosophila melanogaster (Fig. 1A). Although fairly divergent in overall amino acid (aa)2 identity, SNARE proteins share a conserved 60–70-aa SNARE motif, containing a heptad repeat structure (1–3). Key positions in the SNARE motif, including a central zero layer residue, are responsible for the direct interaction between partner SNARE proteins and facilitate the fusion of opposing membranes (Fig. 1, B and C). SNARE proteins are most commonly type II membrane proteins containing a single C-terminal transmembrane domain (TMD), with the majority of the protein exposed to the cytoplasmic side of the membrane (12). A select few SNARE proteins utilize post-translational lipidation, including prenylation and/or palmitoylation, for their membrane attachment (13–17). Even more rare, the SNARE protein SNAP-29 has neither a TMD nor the required cysteine residues for prenylation or palmitoylation and thus has been proposed to associate with the membrane via interaction with other SNAREs (18).

SNARE proteins are functionally classified as either v-SNAREs, which are anchored into transport vesicles, or t-SNAREs, which are tethered to the target compartment (Fig. 1, B and C). When a vesicle docks with its target compartment, fusion of the opposing membranes is mediated by the formation of a SNARE complex, involving a single v-SNARE motif on the vesicle and three t-SNARE motifs on the target membrane (Fig. 1C) (19, 20). The four SNARE motifs wind together, forming a parallel four-helix bundle termed the trans-SNARE complex or SNAREpin (Fig. 1C) (21, 22). Formation of the trans-SNARE complex pulls the opposing bilayers together, providing a mechanical force that directly contributes to the energetics of fusion (23, 24).

Although SNARE proteins are functionally classified as v-SNAREs or t-SNAREs, they are structurally distinguished as

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1 To whom correspondence should be addressed: Depts. of Ophthalmology and Visual Sciences and Genetics and McPherson Eye Research Institute, 600 Highland Ave., Madison, WI 53792. Tel.: 608-265-5398; Fax: 608-265-6021; E-mail: njcolley@wisc.edu.

2 The abbreviations used are: aa, amino acid; ERG, electroretinogram; Endo H, endoglycosidase H; PNGase F, peptide N-glycosidase F; Rh1, rhodopsin; TCH, total cell homogenate; TMD, transmembrane domain; α-Man-II, α-mannosidase-II; ER, endoplasmic reticulum; EMS, ethyl methlysulfonate; Arr, arrestin; TRP, transient receptor potential; TRPL, TRP-like.
R-SNAREs or Q-SNAREs based on the presence of either an arginine (R) or glutamine (Q) residue at the zero layer position of their SNARE motifs (Fig. 1, A and C) (1, 25). Q-SNAREs are further classified into three distinct subgroups, termed Qa, Qb, and Qc (Fig. 1A). According to the SNARE hypothesis, a functional SNARE complex requires one member from each of these four classes of SNARE motifs, such that the zero layer contains one Arg and three Glns (Fig. 1, A and C) (1, 3, 25). A combinatorial use of different SNARE proteins from each subgroup can give rise to a wide array of unique SNARE complexes, each of which functions at a distinct step in the secretory pathway (1).

Gos28 was identified as a 28-kDa Golgi SNARE protein in mammalian cells (26–28) and corresponds to the Gos1p SNARE protein characterized in yeast (29, 30). Numerous studies have shown that Gos28 is evenly distributed across the Golgi, including the cis-, medial-, and trans-cisternae (31–33). Consistent with these findings, Gos28 has been implicated in a variety of transport steps, including anterograde transport between the endoplasmic reticulum (ER) and the Golgi, intra-Golgi transport in both directions, and retrograde transport from the early/recycling endosome back to the trans-Golgi network (28, 29, 32–36). Finally, some of these studies have defined Gos28 as a v-SNARE, whereas some have identified it as a t-SNARE. Therefore, Gos28 may be capable of serving many different functions in the cell.

Here, we characterize the role of Gos28, \textit{in vivo}, for the vesicular transport of rhodopsin (Rh1) through the secretory pathway of \textit{Drosophila} photoreceptor cells. Our results pinpoint a role for Gos28 as a t-SNARE during the trafficking of Rh1 through the distal Golgi compartments. Mutations in \textit{gos28} lead to defective trafficking of Rh1 protein, accumulation of membranes in the secretory pathway, and retinal degeneration. Finally, we have demonstrated that human Gos28 can functionally replace its \textit{Drosophila} homolog and rescue both the Rh1 trafficking defects and the retinal degeneration, showing that Gos28 is conserved from flies to humans.

**EXPERIMENTAL PROCEDURES**

**Genetic Screen and \textit{Drosophila} Strains**—\textit{D. melanogaster} stocks were reared on standard media, on a 12:12 h light/dark cycle at room temperature (22 °C). To isolate the \textit{gos28}\textsuperscript{1}
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mutant, we screened ~12,000 ethyl methylsulfonate (EMS) mutagenized lines from the Zuker collection (37) for the presence or absence of the deep pseudopupil (38). We identified ~900 deep pseudopupil-defective mutants, which were further screened for defects in Rh1 by Western blot analysis (39). The wild-type stocks used in this study were bw;st and cn,bw (parental stocks from the EMS mutagenesis), as well as Canton-S. We also used the eyes absent mutant allele, eya1. The following Bloomington Drosophila Stock Center deficiencies were used for mapping the gos28 EMS mutation: Df(3R)Cha7, Df(3R)-Cha1a, Df(3R)ED2, Df(3R)BX5, and Df(3R)Exel6180. The gos28 P-element allele, P[EPgy2] Gos28EY14669, was generated as part of the gene disruption project by the Berkeley Drosophila Genome Project (40) and was also obtained from the Bloomington Stock Center. Two transgenic stocks, each containing an inducible UAS-RNAi construct directed against the gos28 transcript, P[G(D3051)v12152] and P[K107479]v100289, were obtained from the Vienna Drosophila RNAi Center (41). Eye-specific knockdown of the Gos28 target mRNA transcripts was achieved by performing standard genetic crosses between the UAS-RNAi strains and an eye-specific Gal4 driver line provided by Claude Desplan as follows: P[UAS-Dcr2][P[ey-Gal4], P[GMR-Gal4];+], as described previously (39, 42).

DNA Sequencing—Genomic DNA was isolated from the EMS-mutagenized Drosophila line (gos28') and the wild-type parental line (bw;st) using the DNeasy blood and tissue kit, according to the manufacturer’s instructions (Qiagen Inc., Valencia, CA). The cytological region that we identified by deficiency mapping, 91B5-91C5, corresponds to 30 genes, spanning 177 kb. We designed primer pairs corresponding to genomic animals used in this study, PCR and sequencing were employed to verify the presence of both the gos281 genomic mutation as well as the transgenic copy of gos28.

For heat pulse-chase experiments, we used transgenic flies expressing wild-type Drosophila Rh1 tagged with a 12-amino acid epitope from the C terminus of bovine rhodopsin, P[Rh1-bov]. The epitope tag does not affect Rh1 maturation or function (43). We used transgenic flies expressing the P[Rh1-bov] construct under the control of either the Drosophila hsp70 heat-shock promoter, P[hs-Rh1-bov], or under the control of the endogenous Rh1 promoter, P[ninaE-Rh1-bov] (43). The P[hs-Rh1-bov] construct was expressed in either wild-type or gos281 mutant flies, whereas the P[ninaE-Rh1-bov] construct was expressed in either wild-type or ninaA mutant flies lacking endogenous Rh1 (ninaE127).

Electroretinograms—Electroretinograms were carried out on young adult flies, according to published procedures (44–46). Light generated from a Xenon arc lamp (300 watts) was passed through a monochromator (Oriel Instruments, Stratford, CT), and the data were acquired using the AcqKnowledge III data acquisition system (BIOPAC Systems, Inc., Santa Barbara, CA). Electroretinograms were recorded using 2-s orange or blue light pulses. Orange light intensity (580 nm) was 3 × 10^2 micro-watts/cm^2 and blue light intensity (480 nm) was 2 × 10^2 micro-watts/cm^2.

Western Blot Analysis—Proteins were separated by electrophoresis in SDS-polyacrylamide gels and electroblotted onto nitrocellulose membranes as described previously (47). Antibodies directed to Rh1 (4C5), syntaxin 1A (8C3), β-tubulin (E7), and actin (JLA20-s) were obtained from the Developmental Studies Hybridoma Bank (University of Iowa). Another antibody directed to actin (I-19), as well as an antibody directed to human Gos28 (N-16) were obtained from Santa Cruz Biotechnology. The 1D4 antibody directed to the bovine epiretinae was obtained from P. Robinson, and the CalX antibody was obtained from C. Montell. Antibodies directed to the following proteins were a gift from A. Becker and C. S. Zuker: NinaA, phospholipase C_α (bompa), transient receptor potential (TRP), transient receptor potential-like (TRPL), and arrestin 1 (Arr1). Antibodies directed to calnexin, Arr2, and the G-protein α subunit (G_α) were previously described (38, 48, 49). Finally, polyclonal antibodies directed to Gos28 were generated in rabbits by Cocalico Biologicals (Reamstown, PA) using a full-length Gos28 fusion protein, produced with the pET46 EK/LIC vector and expressed in BL21 (DE3) bacteria with the Novagen Overnight Express Autoinduction System 1 (EMD Biosciences, San Diego). The immunoreactive proteins were visualized using horseradish peroxidase (HRP)-conjugated goat antimouse IgG (Invitrogen), goat anti-rabbit IgG (Invitrogen), don-
key anti-goat IgG (Santa Cruz Biotechnology), or goat anti-mouse IgM (Jackson ImmunoResearch) followed by ECL detection (Thermo Scientific). For quantification, each experiment was repeated a minimum of three times using independent samples, and the blots were re-probed for either β-tubulin or actin as loading controls. In some cases, we loaded more heads per lane for those genotypes that displayed reduced expression. In other cases, we loaded fewer heads per lane to avoid overloading of either the Rh1 band or the Gos28 band. The x-ray film was scanned and subsequently analyzed using ImageJ, version 1.48v (National Institutes of Health, image.nih.gov), to compare the density of the protein bands. The peak densities (total band densities) expressed as a percent of the total of all lanes on the x-ray film were used. For each lane, peak densities were normalized using the peak density of the corresponding loading control. The mean and standard deviations of the three independent experiments were determined. In cases where the quantification involved measuring the relative amount of different molecular weight bands within each lane, loading control data were not necessary. Instead, the relative peak densities, expressed as a percent of the total within the lane, were directly determined in ImageJ. Gels from three independent experiments were analyzed, and the data were expressed as the mean ± S.D. Two-tailed t tests were conducted to determine the p values.

**Immunocytochemistry**—Experiments were carried out on 1–3-day-old flies, as described previously (47). Briefly, heads were fixed in PBS with 3% formaldehyde and 0.01–0.05% glutaraldehyde for 45 min and infiltrated with 2.3 m sucrose overnight. Frozen 0.5–μm sections were immunolabeled with the 4C5 antibody directed to Rh1, obtained from the Developmental Studies Hybridoma Bank (University of Iowa). Primary antibody labeling was detected using DyLight 488-conjugated goat anti-mouse (Jackson ImmunoResearch). Nuclei were labeled with ToPro-3 (Molecular Probes Inc., Eugene, OR). Sections were mounted with Vectashield® (Vector Laboratories, Burlingame, CA) and viewed at room temperature using either a Bio-Rad point-scanning confocal microscope (Radiance 2100 MP Rainbow) or an inverted Nikon Eclipse Ti-E confocal microscope (A1R). In either case, we used a Plan Apo VC ×60, NA 1.40 lens. Imaging data were acquired using either Bio-Rad LaserSharp 6.0 or Nikon Elements 4.13. Color channels were merged using Adobe Photoshop CS6, and panels were arranged in Adobe InDesign CS6. For each experiment, at least three individual heads were sectioned and between 50 and 100 ommatidia were observed in each eye.

**Electron Microscopy**—Adult heads were fixed and processed according to a modification of the methods of Baumann and Walz, as described previously (43, 47). Briefly, heads were fixed in cacodylate buffer containing 2% formaldehyde and 2% glutaraldehyde for 4 h, followed by incubation in the same fixative containing 1% tannic acid overnight at 4 °C. Tissue was rinsed in cacodylate buffer and post-fixed in 2% osmium tetroxide for 2 h. Heads were dehydrated through an ethanol series, rinsed with propylene oxide, and infiltrated and embedded in Spurr’s resin (Electron Microscopy Sciences, Hatfield, PA). Ultrathin sections were stained with 2% uranyl acetate and Reynold’s lead citrate and viewed at 80 kV on a Phillips CM120 electron microscope (University of Wisconsin Medical School Electron Microscope Facility). Images were captured using a MegaView III camera (Olympus Soft Imaging Systems) and iTEM Olympus Soft Imaging Systems (Version 5.0 build 1243) at a magnification of ×1,250–11,500. Image panels were arranged in Adobe InDesign CS6. For each experiment, at least three individual heads were sectioned, and between 50 and 100 ommatidia were observed per eye.

**Biochemical Procedures**—For membrane preparations, wild-type or Gos28<sup>−TMI</sup> mutant fly heads were homogenized into 20 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.2 mM DTT, pH 7.3, containing protease inhibitors. The TCH was sonicated and centrifuged at 100,000 × g for 60 min at 4 °C. The supernatant was mixed with sample buffer (50), and the pellet was resuspended into one of four extraction conditions as follows: 1) hypotonic (low salt) condition, 20 mM Tris, pH 7.3; 2) hypertonic (high salt) condition, 1 mM KCl, 10 mM sodium phosphate, pH 7.3; 3) high pH condition, 100 mM Na<sub>2</sub>CO<sub>3</sub>, pH 11.5; 4) 1% SDS, 20 mM Tris, 150 mM NaCl. All extraction buffers contained 1 mM EDTA and 0.2 mM DTT. The resuspensions were centrifuged at 100,000 × g for 60 min at 4 °C. The supernatant was mixed with sample buffer, and the remaining insoluble material was resuspended into sample buffer. All fractions were assessed via Western blot analysis.

All reagents for the Endo H and PNGase F digestions were obtained from New England Biolabs (Ipswich, MA). For each digestion reaction (or control reaction), 10 wild-type or gos28<sup>f</sup> mutant heads were homogenized into 50 μl of denaturing buffer, sonicated, and processed according to a modification of the manufacturer’s instructions. Specifically, we increased the denaturing buffer to a final concentration of 1% SDS and incubated for 4 h at 22 °C. All samples were mixed with appropriate volumes of sample buffer and assessed via Western blot analysis.

**RESULTS**

**Identification of Mutations in Drosophila gos28**—Flies harboring a mutation in gos28 were identified by screening the Zucker collection of EMS-mutagenized Drosophila (37) for genetic loci that are critical for the proper expression of Rh1 protein. This was accomplished by monitoring the molecular weight of Rh1 via Western blot analysis. We have previously shown that Rh1 is glycosylated in the ER and then completely deglycosylated as it moves through the Golgi, such that defects in Rh1 transport lead to the accumulation of Rh1 in hyperglycosylated high molecular weight forms (39, 47). The gos28<sup>f</sup> EMS allele displayed a defect in Rh1, with a substantial amount of the protein accumulating in a higher molecular weight form, compared with wild type (Fig. 2A). The mutation is recessive, as the heterozygotes displayed normal Rh1 expression (Fig. 2A).

The gos28<sup>f</sup> mutation was localized by deficiency mapping to 91B5–91C5 on the third chromosome (Fig. 2, A and B). We identified a single nucleotide deletion of cytosine 84 in CG7700, encoding Gos28 (Fig. 2B). Drosophila Gos28 displays 43% overall aa identity with human Gos28 (Fig. 2C), encoded by the GOSR1 locus. In both flies and humans, the cytosolic portion of Gos28 contains three putative helical domains termed “a,” “b,” and “c.”

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and “c” followed by a distinct SNARE motif and a single TMD (Fig. 2, B and C).

We obtained a second mutant allele resulting from a P-element insertion between exons 1 and 2, P[EPgy2]Gos28\(^{EY14669}\) (Fig. 2B). As with the EMS allele, the gos28\(^{EY14669}\) homozygotes displayed defects in Rh1 expression, whereas the heterozygotes were wild type (Fig. 2D). The gos28\(^{1}\) and gos28\(^{EY14669}\) mutants are indeed allelic, as the trans-heterozygotes (gos28\(^{1}\) /
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Gos28 is a Membrane Protein—Gos28 was detected as a 24-kDa band in wild-type tissue, was reduced in gos28 homozygotes, and was completely absent in gos28 homozygotes as well as in gos28/gos28^Y14669 trans-heterozygotes (Fig. 3A), demonstrating that Gos28 function is highly conserved from flies to humans.

Drosophila Gos28 Is a Membrane Protein—Gos28 was present exclusively in the membrane pellet following centrifugation of a TCH (Fig. 3D). Gos28 was solubilized by suspension of the membrane pellet in 1% SDS (Fig. 3D), consistent with Gos28 being a membrane protein.

Gos28 Is Required for Effective Rhodopsin Trafficking through the Golgi—In wild-type photoreceptors, Rh1 was precisely localized to the light-sensitive organelles, the rhabdomeres, for its function in phototransduction (Fig. 3E). In gos28 mutants, Rh1 was detected in the rhabdomeres but was also detected in the secretory pathway (Fig. 3E), indicating that there are defects in Rh1 transport. This supports the hypothesis that Gos28 functions in the trafficking of Rh1 during biosynthesis. However, because some Rh1 is properly targeted to the rhabdomeres in gos28 mutants, other SNAREs must compensate for the loss of Gos28.

We also analyzed the kinetics of Rh1 processing by conducting pulse-chase experiments in flies expressing Rh1 under the control of a heat-inducible promoter (43). Following a 1-h heat pulse to initiate Rh1 synthesis, we monitored the transport of Rh1 by assessing its molecular weight changes via Western blot analysis. In wild-type flies, Rh1 was initially detected as glycosylated high molecular weight forms that were processed down to the mature form, beginning ~10 h post heat shock (Fig. 4A) (39, 43). At later time points, Rh1 was detected exclusively in the mature low molecular weight form in wild-type flies.

In gos28^Y14669 mutants, Rh1 was also initially synthesized in the immature high molecular weight form, some of which was processed down to the mature form (Fig. 4B). However, some Rh1 also accumulated in an intermediate form in the gos28 mutants (Fig. 4B). These results indicate that, although the synthesis, glycosylation, and initial trimming of Rh1 proceed normally in the gos28 mutants, the final stages of N-glycan removal are disrupted. Given that Rh1 deglycosylation occurs in the Golgi (39), these results suggest that ER to Golgi transport is not defective in the gos28 mutant, consistent with a role for Gos28 in intra-Golgi transport. It is interesting to note that in both wild-type flies and gos28 mutants, Rh1 levels are severely reduced by 7 days of post-heat shock and undetectable by 10

![FIGURE 2. Identification of Drosophila Gos28. A. Western blot of proteins isolated from 7-day-old fly heads, labeled for Rh1 and re-probed for actin as a loading control. Lanes 1 and 9, wild type (WT, bwst); lane 2, gos28 heterozygotes; lane 3, gos28 homozygotes; lane 4, gos28/Df[3R]Cha7; lane 5, gos28/Df[3R]Cha1; lane 6, gos28/Df[3R]E2D2; lane 7, gos28/Df[3R]X8S; and lane 8, gos28/Df[3R]E6Ex6180. Two heads were loaded per lane. To quantify the amount of Rh1 expressed, the x-ray film was scanned and subsequently analyzed using ImageJ to calculate relative peak densities. Relative peak densities for the loading control were used to normalize the values for Rh1. The means ± S.D. were calculated using the data from three independent experiments. The relative level of Rh1 in each genotype is shown in the upper graph. Each genotype was compared with WT (lane 1), and p values were determined using two-tailed t tests: *, p =0.05; **, p =0.01; ***, p =0.001. The lower graph shows the percentage of Rh1 protein present in the high molecular weight 35-kDa form versus the low molecular weight 34-kDa mature form in each genotype. The means ± S.D. of three independent experiments are shown. The wild-type and gos28 lanes contained extracts from white-eyed flies, whereas all other lanes contained extracts from pigmented eyes. Rh1 protein levels are slightly higher in red-eyed flies compared with white-eyed flies. B, deficiency map showing cytogenetic breakpoints for the deficiencies that failed to complement the gos28^ allele. The gos28 locus is composed of four exons. The red arrow designates the position of the P-element insertion in the drosophila GOS28^ allele, and the red braces indicate the RNAi target region in the GOS28 RNAi alleles. C, alignment between Drosophila (D) and human (H) Gos28 was generated with the UniProt Align program using GenBank accession numbers NP_650739.2 and NP_001007026.1, respectively. Numbers refer to aa residues. Identical aa are indicated by asterisks; strongly similar aa are indicated by colons; and weakly similar aa are indicated by period. D, Western blot of proteins isolated from 7-day-old fly heads, labeled for Rh1 and re-probed for actin as a loading control. Lanes 1 and 12, wild type (WT, bwst); lane 2, gos28 homozygotes; lane 3, gos28^Y14669 homozygotes; lane 4, gos28/gos28^Y14669 trans-heterozygotes; lane 5, gos28/Df[3R]Cha1 RNAi allele; lane 6, gos28^Y107479 RNAi allele; lane 7, gos28 heterozygotes; lane 8, gos28^Y14669 heterozygotes; lane 9, eyes absent (eya) homozygotes; lane 10, P(ina-de-gos28^) (Drosophila Gos28 Rescue), and lane 11, P(ina-de-hGSSR1^) (human Gos28 Rescue). Two heads were loaded per lane, with the exception of lane 2 (four heads), lane 4 (one and a half-heads), lane 7 (one head), and lane 8 (one and a half-heads). The upper graph shows the relative level of Rh1 in each genotype, calculated as described in A. Each genotype was compared with WT (lane 1) and p values are shown: *, p =0.05; **, p =0.01; ***, p =0.001. The lower graph shows the percentage of Rh1 protein present in the high molecular weight 35-kDa form versus the low molecular weight 34-kDa mature form in each genotype. The means ± S.D. of three independent experiments are shown.}
days of post-heat shock (Fig. 4, A and B). These results demonstrate that the time course for Rh1 stability in gos28 mutants is similar to that found in wild-type flies.

To determine more precisely the role of Gos28 in Rh1 transport events, we performed Endo H and PNGase F digestions and assessed shifts in the molecular weight of Rh1. In wild-type flies, the N-glycosylation group is completely removed from Rh1 during its transport to the rhabdomere (39, 47, 51–53). Accordingly, the mature 34-kDa form of Rh1 is insensitive to both Endo H and PNGase F (Fig. 4C). In contrast, treatment of the gos281 mutant tissue with PNGase F shifted the immature high molecular weight band down to the wild-type form (Fig. 4C), confirming that Rh1 was hyperglycosylated in the gos28 mutant. These results are consistent with defects in the transport of Rh1 through the Golgi.

Although Rh1 was hyperglycosylated in the gos281 mutant, the high molecular weight form of Rh1 was insensitive to treatment with Endo H (Fig. 4C). Endo H insensitivity provides insights into the subcellular localization of Rh1 in the gos281 mutant, as Endo H is only able to cleave those N-glycans that

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**FIGURE 3.** Gos28 is a membrane protein required for proper Rh1 localization. A, Western blot of proteins isolated from 2- to 3-day-old fly heads, labeled for Drosophila Gos28, and re-probed for actin as a loading control. Lanes 1 and 12, wild type (WT, bw;st); lane 2, gos281 homozygotes; lane 3, gos281 homozygotes; lane 4, gos281 homozygotes; lane 6, gos28EY14669 RNAi allele; lane 7, gos281 heterozygotes; lane 8, gos28EY14669 RNAi allele; lane 9, eyes absent (eya1) homozygotes; lane 10, P(ninaE-gos281) [Drosophila Gos28 Rescue]; and lane 11, wild type. Each genotype was compared with WT (lane 1) and p values are shown: *, p < 0.05; **, p < 0.01; ***, p < 0.001.

B, Western blot of proteins isolated from 2- to 3-day-old fly heads, labeled for human Gos28, and re-probed for actin as a loading control. Lane 1, human retina control; lane 2, P(ninaE-hGOSR1/H11001) (human Gos28 Rescue); lane 3, gos281 homozygotes, and lane 4, wild type (WT, bw;st). 35 fly heads were loaded per lane (lanes 2–4). C, hydrophathy plot of the Drosophila Gos28 protein, analyzed by the Kyte-Doolittle algorithm. A single hydrophobic region (black shading) corresponds to the TMD. D, Western blot of protein fractions generated from 0- to 7-day-old wild type (Canton-S) heads following differential centrifugation of a TCH, labeled for Drosophila Gos28 and re-probed for arrestin 2 as a loading control. S, supernatant; P, pellet, generated from the membrane-bound fraction. E, confocal images of 0.5–2 mm cross-sections from wild type (WT, cn,bw) and gos281 mutant heads, labeled for Rh1 (green). Nuclei were stained with ToPro3 (blue). White arrows indicate secretory pathway labeling in the mutant. A schematic of a cross-section from the R1–7 photoreceptor cells is shown. The rhabdomeres of the R1–6 photoreceptors (R, green), secretory pathway membrane system (black), and nuclei (N, blue) are shown for reference. In left panels, scale bars equal 2 μm. In middle and right panels, scale bars equal 1 μm.

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have not yet been processed by α-mannosidase-II (α-Man-II), a glycosyl hydrolase enzyme residing in the Golgi (39, 54, 55). Thus, glycoproteins that are sensitive to Endo H digestion typically reside in the ER, whereas glycoproteins that are insensitive to Endo H have successfully reached the Golgi and been processed by α-Man-II. Although the distribution of α-Man-II may vary slightly from one cell type to another, α-Man-II is predominantly localized to the medial- and/or trans-cisternae of the Golgi (56). Therefore, the finding that the Rh1 protein was insensitive to Endo H treatment in the gos28′ mutant indicates that Rh1 had successfully reached the medial-Golgi compartment where α-Man-II resides (Fig. 4D).

Finally, the finding that some Rh1 was abnormally hyperglycosylated in the gos28′ mutant suggests that the Rh1 protein failed to effectively navigate the remainder of the secretory pathway. Rh1 is unique among glycoproteins in that the N-gly-
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can be completely removed from Rh1 during its transport through the cell by a cascade of enzymes (39). Therefore, the presence of the hyperglycosylated form of Rh1 in the gos28/ mutant indicates that the protein had not been properly processed by the glycosyl hydrolases that function downstream from a-Man-II and are responsible for trimming the remaining mannose residues from the N-glycan (39). Given that these downstream enzymes localize to the Golgi, our results indicate that Rh1 does not properly exit the Golgi in the gos28/ mutant. Taken together, our data suggest that Gos28 functions in medial- to trans-Golgi transport of Rh1 protein during biosynthesis (Fig. 4D).

Gos28 Is Uniquely Required by Rh1 in Photoreceptor Cells—Despite the ubiquitous expression of Gos28 during development and in the adult (modmECODE and FlyAtlas (57)), the only discernable defects in the gos28 mutants were in the eye. To determine more precisely the requirements for Gos28 in the eye, we examined the expression of photoreceptor cell proteins that are critical for phototransduction and/or photoreceptor cell integrity. The G-protein α subunit (Gα), the no-receptor-potential A (norpA)-encoded phospholipase C effector molecule (PLCβ), the TRP and TRPL cation-selective channels, as well as Arr1 and Arr2, were all expressed at normal levels in the gos28/ and gos28/EY14669 mutants (Fig. 5A). Other photoreceptor cell proteins, including NiNa, calnexin, and the Na+/Ca2+ exchanger (CalX), were also expressed at wild-type levels in the gos28 mutants (Fig. 5A). Taken together, these results indicate that, within photoreceptor cells, Gos28 is uniquely required for the proper expression of Rh1.

We also performed electroretinogram analysis to determine whether Rh1 and the phototransduction cascade were functional in gos28 mutants. We found that the light response was normal in gos28/ mutant photoreceptor cells (Fig. 5B). Additionally, the gos28/ mutants demonstrated a prolonged depolarizing after-potential in response to blue light stimulation (Fig. 5B). These results indicate that at least 20% of the Rh1 was functional (58), and that sufficient amounts of the other phototransduction proteins were also present and functional in the rhahdomeres. The electroretinogram traces were also used to determine whether Gos28 plays a role in synaptic transmission between the photoreceptor cells and their second order neurons in the brain. Functional neurotransmission can be monitored by the presence of on- and off-transients (45, 59). Both of these features were normal in the gos28/ mutant (Fig. 5B), indicating that Gos28 is not required for neurotransmitter release at the synapse and consistent with its predicted role in the Golgi.

Mutations in gos28 Lead to Retinal Degeneration—Defects in photoreceptor cell morphology were observed in young gos28 mutants, compared with wild-type (Fig. 6A). There was substantial vacuolation of the cells and, at higher magnification, enlarged ER and Golgi membranes were observed (Fig. 6A). These results are consistent with the accumulation of Rh1 in the secretory pathway of gos28 mutants (Fig. 3E), as we have previously shown that defects in Rh1 trafficking lead to the overproliferation of ER and Golgi membranes (38, 43, 47, 49, 53, 60). By 1 month of age, the vacuolated tissue was significantly more prominent in the gos28 mutants, and in many cases, entire clusters of photoreceptor cells were missing (Fig. 6A). These data demonstrate that mutations in gos28 lead to retinal degeneration, indicating that Gos28 is essential for photoreceptor cell integrity.

Introduction of a wild-type copy of Drosophila gos28 into the genome of the gos28/ mutant prevented the retinal degeneration (Fig. 6B, Dros Rescue). These results are consistent with the restoration of wild-type Rh1 expression in the Drosophila Gos28 rescue flies (Fig. 2D) and demonstrate that gos28 is indeed the locus responsible for the mutant phenotype. Introduction of wild-type human Gos28 also rescued the retinal degeneration in the gos28/ mutant flies (Fig. 6B, Human Rescue). These results are consistent with the rescue of Rh1 transport in the human rescue flies (Fig. 2D) and, once again, demonstrate the functional conservation between human and Drosophila Gos28.

Zero Layer Is Required for Gos28 Function—Consistent with their assignment to the Qb family of t-SNAREs, both human and Drosophila Gos28 harbor a glutamine residue at the zero layer. However, both proteins also contain an arginine residue directly to the right (Fig. 7A). This “QR” zero layer composition is not unique to Gos28, as other members of the Qb subfamily of t-SNAREs also have this sequence. It is possible that the Arg lies sufficiently close to the zero layer to mediate interactions with three Q-SNARE motifs, thereby allowing these proteins to function as R-SNAREs. To determine the functional significance of the Gln and the Arg in the SNARE motif, we conducted site-di-
rected mutagenesis and produced transgenic animals harboring amino acid substitutions in Gos28 (Fig. 7A). First, we expressed the transgenes in gos28' mutant flies (Fig. 7B) to assess whether the altered Gos28 proteins were functional and thus able to mediate normal Rh1 trafficking (Fig. 7C). Second, we expressed the transgenes in wild-type flies (Fig. 7D) to assess
their potential dominant negative effects on Rh1 (Fig. 7E). In both cases, expression of the transgenic protein was confirmed by Western blot analysis (Fig. 7, B and D), and the molecular weight of Rh1 was monitored to determine Gos28 function (Fig. 7, C and E).

In the first mutant, Gos28Q176G/R177G, we replaced both the zero layer Gln and the neighboring Arg with glycine residues to determine whether either of these amino acids were critical for Gos28 function during Rh1 transport. In the second mutant, Gos28Q176G, we replaced the zero layer Gln with a Gly, leaving the Arg intact, to determine whether the Gln was necessary for Rh1 transport or whether the Arg might be sufficient. In the third mutant, Gos28R177G, we replaced the Arg with a Gly, this time leaving the zero layer intact, to determine whether the Arg was necessary for Rh1 transport or whether the zero layer Gln was sufficient. In the fourth mutant, Gos28Q176R/R177G, we artificially shifted the Arg into the zero layer position. Finally, in the fifth mutant, Gos28Q176E, we replaced the zero layer Gln residue with a negatively charged glutamic acid residue. In all five cases, the mutant Gos28 proteins were unable to mediate normal Rh1 expression, as Rh1 was detected in a high molecular weight form (Fig. 7C). These results demonstrate that Gln-176...
and Arg-177 are both required for Gos28 function in vivo, but neither residue on its own is sufficient for function.

To further investigate the functional significance of the zero layer residues, the Gos28Q176G/R177G, Gos28Q176G, Gos28R177G, Gos28Q176R/R177G, and Gos28Q176E mutant transgenes were expressed in wild-type flies, which also expressed endogenous Gos28. All five mutants displayed Rh1 in a high molecular weight form (Fig. 7E) demonstrating that the mutant forms of Gos28 exert a dominant negative effect. It is important to note that expression of the wild-type Gos28 rescue transgene did not have a dominant negative effect, as Rh1 protein was wild type in these flies (Fig. 7E). Considering the nature of SNARE-SNARE
interactions during vesicle fusion (Fig. 1C), a dominant negative effect could be explained by the mutant forms of Gos28 competing with wild-type Gos28 for SNARE protein interactions. If these interactions were nonfunctional, then the mutant forms of Gos28 would sequester their interacting SNAREs into dead-end SNARE complexes, incapable of facilitating vesicle fusion.

**Gos28 Functions as a t-SNARE for Rh1 Transport**—Most SNARE proteins are membrane-anchored via a single C-terminal TMD. The TMD plays an essential role in membrane fusion by coupling the lipid bilayer to the four-helix bundle, thereby transmitting the energy of the SNARE complex assembly to the adjacent membranes (3, 61). Importantly, in order for SNARE-mediated membrane fusion to occur, at least one SNARE protein in the trans-SNARE complex must be anchored into each of the opposing membranes (62, 63). For this reason, v-SNAREs require functional TMDs to facilitate membrane fusion, whereas t-SNAREs can rely on the TMD of one or more of their partner SNAREs and thus have more flexibility with regard to their method of membrane association (63–66). To determine whether the TMD was critical for Gos28 function in vivo, we generated transgenic flies expressing Gos28 lacking its C-terminal TMD (Gos28−TMD). As predicted, the Gos28−TMD protein was expressed as a lower molecular weight band (Fig. 7, B and D). Ingos28−TMD mutant transgenic flies, this was the only form of Gos28 detected (Fig. 7B), whereas in transgenic wild-type flies, the Gos28−TMD protein was detected directly below the endogenous, full-length copy of Gos28 (Fig. 7D).

Expression of the Gos28−TMD protein was able to rescue the Rh1 defects observed in the gox28−TMD mutant flies (Fig. 7C). Rh1 expression was also normal in wild-type flies expressing the Gos28−TMD construct (Fig. 7E), indicating that the Gos28−TMD protein does not exert a dominant negative effect during Rh1 transport. Furthermore, expression of Gos28−TMD rescued the retinal degeneration in the gox28−TMD mutant flies (Fig. 6B). Taken together, these data demonstrate that, for Gos28, the TMD is not required for functional SNARE complex assembly and that the N terminus (containing the SNARE motif) is sufficient to facilitate Rh1 transport.

To determine whether the Gos28−TMD protein was soluble, we performed centrifugation of a TCH generated from transgenic wild-type flies that expressed the Gos28−TMD protein. In these flies, both the endogenous Gos28 protein and the truncated Gos28−TMD protein were expressed, allowing us to visualize both proteins simultaneously (Fig. 8, A–D). As with the wild-type Gos28 protein, Gos28−TMD was present exclusively in the membrane pellet (Fig. 8, A–D, lanes 1–3) indicating that, despite the absence of a TMD, the Gos28−TMD protein is tightly associated with the membrane. Interestingly, Gos28 lacks a consensus sequence for prenylation, palmitoylation, myristoylation, or glycosyrophosphatidylinositol anchor attachment. Furthermore, there are only seven, mainly charged amino acids located C-terminal to the SNARE motif in the Gos28−TMD mutant, making lipid modification unlikely.

It has been suggested that certain t-SNAREs can function in the absence of either a TMD or a post-translational lipid modification, presumably due to their association with other membrane-bound t-SNAREs (17, 18, 64, 67). To determine whether the Gos28−TMD protein was associated with the membrane via protein-protein interactions, we first examined the sensitivity of the membrane association by either a low salt (hypotonic) or high salt (hypertonic) extraction, both of which act to alter the strength of ionic (or polar) interactions between proteins (68, 69). Fig. 8, A and B, shows that the Gos28−TMD mutant’s association with the membrane was insensitive to low salt or high salt extraction, as the protein was detected solely in the membrane pellet following both treatments. These results suggest that the Gos28−TMD protein is not associated with the membrane via predominantly polar or protein-protein interactions, but rather it uses some other type of interaction. Interestingly, most of the binding energy of the four-helix SNARE complex comes from strong hydrophobic interactions facilitated by a series of highly conserved amino acids that lie along the SNARE motif (1, 22). Therefore, if the Gos28−TMD protein was bound to the membrane via its association with partner SNARE proteins, one would not expect this mostly hydrophobic interaction to be disrupted by simply altering the ionic strength of the solution. Results obtained for the Gos28−TMD protein are in contrast to those obtained for Arr2, a well known peripheral membrane protein (70, 71). A small amount of Arr2 was liberated from the membrane following low salt extraction (Fig. 8A), whereas a more substantial amount of Arr2 was liberated following high salt extraction (Fig. 8B). Other controls included the integral membrane proteins, syntaxin 1A and Rh1, both of which were insensitive to low or high salt extraction (Fig. 8, A and B).

We further examined the sensitivity of the Gos28−TMD protein’s membrane association by extracting with high pH. Using a sodium carbonate treatment, closed membranes are converted into open membrane sheets, liberating content proteins as well as peripheral membrane proteins (68, 72). Fig. 8C shows that the Gos28−TMD protein was successfully extracted from the membrane with high pH, as a substantial amount of the Gos28−TMD protein was detected in the supernatant fraction. Given that the Gos28−TMD protein was absent from the initial soluble fraction, these data are consistent with Gos28 being a peripheral membrane protein. Efficient extraction was also observed for the peripheral membrane protein, Arr2 (Fig. 8C). In contrast, only small amounts of the wild-type Gos28 protein and the syntaxin 1A SNARE protein were extracted using high pH, whereas Rh1 protein, which contains seven TMDs, was completely insensitive to extraction with high pH (Fig. 8C). Finally, all proteins were readily extracted from the membrane fraction using 1% SDS (Fig. 8D). Taken together, these results indicate that the Gos28−TMD protein is tethered to the membrane via interactions with other proteins. Furthermore, insensitivity to extraction with high salt, but sensitivity to extraction with high pH and 1% SDS, are consistent with the properties associated with SNARE-SNARE interactions.

It has been shown in several instances that SNARE proteins can form homotypic interactions (73–77). These interactions typically involve the TMD, which was clearly absent in the Gos28−TMD protein. Nonetheless, it remained possible that the Gos28−TMD protein was associated with the membrane via interaction with endogenous Gos28. To rule out this possibility,
we performed biochemical fractionation of a TCH generated from gos28 \textsuperscript{-} mutant flies that expressed the Gos28 \textsuperscript{−TMD} protein. In these flies, the endogenous wild-type Gos28 protein was absent, and only the truncated Gos28 \textsuperscript{−TMD} protein was expressed (Fig. 8E). As before, the Gos28 \textsuperscript{−TMD} protein was present almost exclusively in the membrane pellet (Fig. 8E). That the Gos28 \textsuperscript{−TMD} protein was still associated with the membrane in the absence of the wild-type Gos28 protein demonstrates that the membrane association was not facilitated via homotypic interactions with wild-type Gos28. Therefore, we propose that the Gos28 \textsuperscript{−TMD} protein is interacting with its partner SNARE proteins in the target membrane. Although this represents a plausible mechanism for a t-SNARE, it would be impossible for a lone v-SNARE to function without some form of membrane attachment (Fig. 8F). The finding that the Gos28 \textsuperscript{−TMD} protein is functional for Rh1 transport supports the concept that Gos28 functions as a t-SNARE for Rh1 transport through the distal compartments of the Golgi.

**DISCUSSION**

Here, we characterize the role of Drosophila Gos28, in vivo, and demonstrate that mutations in gos28 lead to defects in Rh1 trafficking and retinal degeneration. We show that null alleles of gos28 are homozygous-viable in Drosophila. Consistent with our findings, genetic elimination of gos28 in both Saccharomyces cerevisiae (29) and Caenorhabditis elegans (78) yielded viable organisms. Taken together, these studies demonstrate that, although Gos28 is ubiquitously expressed, other SNARE pro-
teins with redundant functions likely compensate for the loss of Gos28 in most instances. Indeed, it was demonstrated in C. elegans that Gos28 and Ykt6 act redundantly during embryonic development and for the proper expression of Golgi-resident proteins in the adult (78).

Consistent with redundancy among SNAREs, we have shown in Drosophila that Gos28 is specifically required for the proper expression of Rh1 protein, but not for other photoreceptor cell proteins. Furthermore, in the absence of Gos28, significant amounts of Rh1 still traffic to the rhabdomere. These results indicate that more than one SNARE complex must be capable of facilitating the transport of Rh1 through the Golgi. This is not the first example of two SNARE complexes acting simultaneously and with similar kinetics for a single transport step. Two completely distinct SNARE complexes are required for retrograde trafficking from the endosome to the trans-Golgi network (79). Furthermore, it was shown that if one of these SNARE complexes is disrupted, this transport process is only partially disrupted, demonstrating that both complexes are required and yet neither complex is sufficient. A similar mechanism may be occurring in Drosophila for Rh1 transport through the Golgi.

In the absence of Gos28, a substantial amount of Rh1 protein accumulated in an immature high molecular weight form. In addition, point mutations altering the SNARE domain in Gos28 led to accumulations of the immature form of Rh1. These defects were detected even when the mutant Gos28 proteins were expressed in the presence of wild-type Gos28. This dominant negative effect could be explained by the mutant form of Gos28 interacting with its partner SNARE proteins and inhibiting their ability to mediate membrane fusion. Inhibitory interactions between Gos28 and its binding partners could be particularly deleterious, given that SNARE proteins can participate in multiple transport events, each invoking a unique set of SNARE partners.

To date, Gos28 has been isolated in three distinct SNARE complexes as follows: one with Ykt6, syntaxin 5, and Bet1 (36); a second with Ykt6, syntaxin 5, and Gos15 (23, 35, 79, 80); and a third with Sec22b, syntaxin 5, and Bet1 (34). This complexity is compounded by the fact that each of Gos28’s interacting partners may also be involved in additional SNARE complexes that do not involve Gos28. Therefore, expressing mutant forms of Gos28 may not only disrupt SNARE complexes that normally involve Gos28, but they could also sequester Gos28’s interacting partners away from additional SNARE complexes that function for Rh1 transport. Competitive inhibition would explain why mutations in Gos28’s SNARE motif cause more severe defects in Rh1 transport than null mutations in gos28.

Consistent with its participation in numerous different SNARE complexes, Gos28 has been implicated in a wide variety of vesicular trafficking events, including anterograde transport from the ER to the Golgi, intra-Golgi movement in both the anterograde and retrograde directions, and finally retrograde transport from the endosome back to the trans-Golgi network. Given its widespread expression throughout the cis-, medial-, and trans-Golgi compartments, it is possible that Gos28 plays a role in all of these transport events. However, the in vivo functions of Gos28 in specific transport events are largely unknown. In this study, we have identified an essential role for Gos28 in Rh1 trafficking through Drosophila photoreceptor cells. Gos28 does not appear to be required for ER to Golgi transport of Rh1, but rather it plays a critical role in downstream trafficking steps, most likely from the medial- to trans-compartments of the Golgi.

There has been debate about the role of Gos28 as either a v-SNARE or a t-SNARE. Here, we show that in the absence of its TMD, Gos28 is still capable of facilitating membrane fusion, likely via its interaction with other membrane-bound SNARE proteins. Indeed, several SNARE proteins are attached to the membrane by post-translational modifications, such as prenylation and/or palmitoylation (13–17, 81–83). However, Gos28 has no consensus sequences for prenylation, palmitoylation, or any other lipid modifications such as myristoylation or glycosylphosphatidylinositol anchor attachment. Although the amino acid sequence requirements for palmitoylation are not well defined, it has been thoroughly established that the acylated cysteine residue is always located at the C terminus of the SNARE motif, either in place of a TMD, within a TMD, or immediately adjacent to a TMD (13–15, 81–83). The only cysteine residue that remains on the Gos28 TMD protein is located at the N terminus of the SNARE motif (Fig. 2C); thus, palmitoylation of this residue would render a topology that is incompatible for SNARE complex formation. Finally, it is important to note that these types of lipid modifications, when present on SNARE proteins, are typically insufficient to facilitate membrane fusion if the protein functions as a v-SNARE. Numerous studies have shown that lipid anchors cannot functionally replace TMDs, suggesting that a full-length membrane anchor spanning the lipid bilayer is a prerequisite for vesicle fusion (62, 63, 65, 66, 75). Taken together, our findings are consistent with a role for Gos28 as a t-SNARE for membrane fusion of Rh1-containing vesicles, because a v-SNARE lacking a TMD would be unable to mediate membrane fusion, even with the aid of a lipid anchor. Our findings are consistent with liposome experiments in yeast, demonstrating that Gos1p (Gos28), Sed5 (syntaxin 5), Ykt6, and Sft1 (Gos15) mediate membrane fusion in a topologically restricted manner (23). Specifically, the authors showed that Gos28 must be positioned on the target membrane to facilitate fusion, again suggesting that Gos28 functions as a t-SNARE.

Because defects in protein trafficking are inextricably linked to human disease and because SNARE proteins constitute a universal machinery required for membrane fusion in eukaryotes, understanding their function in Drosophila provides broad, fundamental insights into mechanisms underlying various human diseases. Here, we show that mutations in gos28 lead to defects in the intra-Golgi transport of Rh1 and lead to retinal degeneration. Furthermore, we have demonstrated that the human Gos28 SNARE protein can functionally replace the Drosophila homolog and rescue both the Rh1 trafficking defects and the retinal degeneration. Our results identify Gos28 as an essential component of the protein trafficking machinery in Drosophila photoreceptor cells and provide mechanistic insights into the role of SNARE proteins in neurodegenerative disease.
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