Characterization of SNAREs Determines the Absence of a Typical Golgi Apparatus in the Ancient Eukaryote *Giardia lamblia*§

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*Giardia* is a eukaryotic protozoal parasite with unusual characteristics, such as the absence of a morphologically evident Golgi apparatus. Although both constitutive and regulated pathways for protein secretion are evident in *Giardia*, little is known about the mechanisms involved in vesicular docking and fusion. In higher eukaryotes, soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) of the vesicle-associated membrane protein and syntaxin families play essential roles in these processes. In this work we identified and characterized genes for 17 SNAREs in *Giardia* to define the minimal set of subcellular organelles present during growth and encystation, in particular the presence or not of a Golgi apparatus. Expression and localization of all *Giardia* SNAREs demonstrate their presence in distinct subcellular compartments, which may represent the extent of the endomembrane system in eukaryotes. Remarkably, *Giardia* SNAREs, homologous to Golgi SNAREs from other organisms, do not allow the detection of a typical Golgi apparatus in either proliferating or differentiating trophozoites. However, some features of the Golgi, such as the packaging and sorting function, seem to be performed by the endoplasmic reticulum and/or the nuclear envelope. Moreover, depletion of individual genes demonstrated that several SNAREs are essential for viability, whereas others are dispensable. Thus, *Giardia* requires a smaller number of SNAREs compared with other eukaryotes to accomplish all of the vesicle trafficking events that are critical for the growth and differentiation of this important human pathogen.

*Giardia*, which is considered one of the most ancient eukaryotes, is a flagellated, biflagellated protozoan that parasitizes the upper small intestine of an extensive variety of vertebrate hosts (1). Human infections are caused by *Giardia lamblia*, the most commonly reported intestinal parasite in the world. *Giardia* has a simple life cycle alternating between disease-causing trophozoites and environmentally resistant cysts, which are responsible for transmission of the parasite among susceptible hosts (2).

The typical Golgi apparatus of most eukaryotic organisms consists of a number of flattened cisternae arranged in a stack, which functions as the protein delivery center of the cell and performs multiple modifications of lipids and proteins (3). *G. lamblia* trophozoites possess an interesting secretory system in which a morphologically identifiable Golgi apparatus seems to be absent, although the packaging and sorting functions of this organelle are obvious in this organism (4, 5). For example, transport to the plasma membrane (PM) and release into the culture medium of variant-specific surface proteins (VSPs) (6, 7), as well as trafficking of both membrane and soluble enzymes to peripheral vacuoles (PVs), which are thought to perform both lysosomal and endosomal activities (4, 8), are evidence for constitutive protein transport. Regulated secretion has been reported to take place only during trophozoite differentiation into cysts (9). Encystation can be reproduced in the laboratory by replacing the culture medium with an encystation medium containing high concentrations of bile and a pH resembling that of the small intestine (10). By using this *in vitro* system, it was found that cyst formation comprises different steps that include the expression of encystation-specific genes, such as those necessary for the synthesis and processing of cyst wall components (11–13) as well as the biogenesis of electron-dense secretory granules (encystation-specific secretory vesicles (ESVs)) that transport cyst wall material to the cell periphery (11, 12). Before exocytosis, the ESVs interact with the PVs, a

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1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S10 and Tables S1–S5.

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2 The abbreviations used are: PM, plasma membrane; ER, endoplasmic reticulum; ESV, encystation-specific secretory vesicle; PV, peripheral vacuole; HA, hemagglutinin; FITC, fluorescein isothiocyanate; IFA, immunofluorescence assay; mAb, monoclonal antibody; CWP, cyst wall protein; VSP, variant-specific surface protein; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; gSNARE, *Giardia* SNARE; VAMP, vesicle-associated membrane proteins; ORF, open reading frame; HMW, high molecular weight; TGN, trans-Golgi network.
SNAREs Define Vesicular Compartments in Giardia

Construction of Expression Vectors—For expression and localization of SNARE proteins in Giardia, the entire open reading frame of each SNARE gene was amplified by PCR with the following sense oligonucleotides containing BamHI or BglII sites and antisense oligonucleotides containing a Ncol site: gQa1_f, 5′-GCG GAT CCG AGA ACA TGT ATG ACG AC-3′; gQa1_r, 5′-ATA AGA ATG CGG CCG CTC AGT TGA TAT-3′; gQa2_f, 5′-GCG GAT CCA CAG TGA CCA-3′; gQa2_r, 5′-ATA AGA ATG CGG CCG-3′; gQa3_f, 5′-GAA GAT CTA CCG ATT TTG ATG CAC CA-3′; gQa3_r, 5′-ATA AGA ATG CGG CCG CCG CCT ATA TAA GTC GCA GAA A-3′; gQa4_f, 5′-GCG GAT CCT CTG ACT TTG ATG ACC CAC-3′; gQa4_r, 5′-ATA AGA ATG CGG CCG CCT ATA GAA GAT-3′; gQa5_f, 5′-GCG GAT CCT CTG ACT TTG ATG ACC CAC CAC-3′; gQa5_r, 5′-ATA AGA ATG CGG CCG CCT ATA GAA GAT-3′.

SNARE-mediated fusion typically results from the formation of a complex that comprises a supercoil of four α-helical coils, consisting of one R-SNARE and three Q-SNARE motifs (26). In this context, Q-SNAREs can be subdivided into Qa-SNAREs (syntaxins), Qb-SNAREs (25-kDa synaptosomal-associated protein (SNAP-25) N-terminal SNARE motif), and Qc-SNAREs (SNAP-25 C-terminal SNARE motif) (30). The R-SNAREs also can be subdivided into short VAMPS (vesicle-associated membrane proteins), or “brevins” (e.g. synapto-brevin), and long VAMPS, or “longins,” on the basis of whether they contain a short and variable domain or a conserved long domain of 120–140 amino acids at their N termini (31). SNARE complexes may be assembled from three or four proteins (SNAP-25 homologs typically contribute two helices), and the complete complex is a four-helix bundle composed of one helix from each sequence family, Qa, Qb, Qc, and R (24).

Like Q-SNAREs, R-SNAREs can include transmembrane domains or can be covalently anchored to a lipid at conserved cysteine residues (31). Indeed, a phylogenetic analysis of SNARE sequences from Saccharomyces cerevisiae, Arabidopsis thaliana, some protozoa, and mammals shows that these four SNARE subfamilies are highly conserved and diverged early in eukaryotic evolution (21).

Because Giardia is an early divergent eukaryote (1, 2), the study of the organization of membrane compartments and membrane fusion processes in this organism may allow a better understanding of the evolution of transport pathways from primitive to higher eukaryotes. In addition, because of their parasitic life style, Giardia is an interesting system in which to analyze how microorganisms could have lost complex organelles and, therefore, to illuminate the minimal set of organelles needed for a given cellular function. For these reasons, the aim of the present work was to identify and characterize the complete set of SNARE proteins in Giardia to define the minimal set of subcellular compartments present during growth and encystation, in particular the existence or not of a typical Golgi apparatus. Our results suggest that gSNAREs are functional and distinctively localized to known subcellular compartments, suggesting a role in regulating membrane trafficking to and from these organelles. Surprisingly, a comparison of Giardia SNAREs homologous to Golgi SNAREs from other organisms does not allow the detection of a typical Golgi apparatus in either proliferating or differentiating trophozoites.

EXPERIMENTAL PROCEDURES
SNAREs Define Vesicular Compartments in Giardia

CTG ATT CGG G-3'; gQb3_r, 5'-ATA AGA ATG CGG CGG CCT TTT AAC GAA AAA ACG GGG GA-3'; gQb4_f, 5'-CGC GGA TCC GAA AGA GAT TGT TCT ATG G-3'; gQb4_r, 5'-ATA AGA ATG CGG CGG CCT TTC TCT AAT ATC GTA TGA TCT CGT GCT AGG GCC GC-3'; gQb5_f, 5'-CGC GGA TCC GAA AGA GAT TGT TCT ATG G-3'; gQc1_f, 5'-CGC GGA TCC TTC TTG GGT GCC GAT G-3';

gQc1_r, 5'-ATA AGA ATG CGG CGG CCT TTC AAC CAG TGA TTT TAG CAA T-3'; gQc2_f, 5'-CGC GGA TCC TTC TTG GGT GCC GAT G-3'; gQc2_r, 5'-ATA AGA ATG CGG CGG CCT TTC AAC CAG TGA TTT TAG CAA T-3'; gQa2A_r, 5'-ATA AGA ATG CGG CGG CCT TTC AAC CAG TGA TTT TAG CAA T-3'; gQa2A_f, 5'-ATA AGA ATG CGG CGG CCT TTC AAC CAG TGA TTT TAG CAA T-3'; gQb1A_r, 5'-ATA AGA ATG CGG CGG CCT TTC AAC CAG TGA TTT TAG CAA T-3'; gQb1A_f, 5'-ATA AGA ATG CGG CGG CCT TTC AAC CAG TGA TTT TAG CAA T-3'; gQa5A_f, 5'-CGC GGA TCC TTC TTG GGT GCC GAT G-3'; gQa5A_r, 5'-ATA AGA ATG CGG CGG CCT TTC AAC CAG TGA TTT TAG CAA T-3'; gQa5A_r, 5'-ATA AGA ATG CGG CGG CCT TTC AAC CAG TGA TTT TAG CAA T-3'; gQb5A_f, 5'-CGC GGA TCC TTC TTG GGT GCC GAT G-3'; gQb5A_r, 5'-ATA AGA ATG CGG CGG CCT TTC AAC CAG TGA TTT TAG CAA T-3'; gQb5A_r, 5'-ATA AGA ATG CGG CGG CCT TTC AAC CAG TGA TTT TAG CAA T-3';

gQb1A_f, 5'-ATA AGA ATG CGG CGG CCT TTC AAC CAG TGA TTT TAG CAA T-3'; gQb1A_r, 5'-ATA AGA ATG CGG CGG CCT TTC AAC CAG TGA TTT TAG CAA T-3'; gQb1A_f, 5'-ATA AGA ATG CGG CGG CCT TTC AAC CAG TGA TTT TAG CAA T-3'; gQb1A_r, 5'-ATA AGA ATG CGG CGG CCT TTC AAC CAG TGA TTT TAG CAA T-3'; gQb5A_r, 5'-ATA AGA ATG CGG CGG CCT TTC AAC CAG TGA TTT TAG CAA T-3'; gQb5A_r, 5'-ATA AGA ATG CGG CGG CCT TTC AAC CAG TGA TTT TAG CAA T-3';

PCR products were purified, cut, and cloned into the vector pTubHAmap N terminus (4). In this way, each SNARE gene was inserted inside the pTubHAmap N terminus inversely, thus generating each SNARE antisense vector. Sequences were confirmed by dye terminator cycle sequencing (Beckman Coulter). Transfection of G. lamblia trophozoites was performed by electroporation as described previously (32), and cells carrying the antisense construct were selected using puromycin (33).

G. lamblia Cultivation and Transfection—Trophozoites of the isolate clone WB/1267 (34) were cultured as described previously (35). Encystation of the trophozoite monolayer was accomplished by the method described by Boucher and Gillin (36). Trophozoites were transfected with the constructs by electroporation and selected with puromycin as described previously (37). After transfection, clones with low expression of gSNARE proteins were selected by immunoblotting and IFA.

Immunofluorescence Analysis of Giardia Trophozoites—Cells cultured in either growth or encystation medium were harvested and processed as described previously (12). The cells were fixed with 4% paraformaldehyde and permeabilized for 1 h at room temperature in phosphate-buffered saline, 0.1% Triton X-100, 10% goat serum. The cells then were incubated with the antibodies diluted in phosphate-buffered saline, 0.1% Triton X-100, 3% goat serum. For indirect staining, slides were incubated with the specific mAbs (final dilution 1:200) or anti-hemagglutinin (HA) mAb (Sigma-Aldrich, final dilution 1:1000) for 1 h at 37 °C and then with anti-mouse secondary antibody labeled with FITC (final dilution 1:250) for 1 h at 37 °C. For direct double staining, anti-HA mAb FITC (Sigma-Aldrich, final dilution 1:500) was used to detect the transgenic proteins; 9C9, 5B11, 5C1, and 7D2 mAbs were labeled directly with Texas Red (Zenon One, Molecular Probes, Inc., Eugene, OR) for the detection of BiP, PVs, VSP1267, and the endogenous
CWP2, respectively. Controls included the omission of primary antibodies and staining of cells transfected with the empty vector. Confocal images were collected using a Zeiss LSM 5 Pascal laser-scanning confocal microscope equipped with an argon/helium/neon laser and an ×100 1.4 NA oil immersion objective (Zeiss Plan-Apochromat). Single confocal sections (0.3 μm) were taken parallel to the coverslip (xy sections). Images were acquired and processed with LSM software; blind two-dimensional deconvolution was performed with AutoDeblur & Auto-Visualize Gold.

**Nucleic Acid Analysis**—Genomic DNA used for the PCRs was obtained as described (7). To determine whether the genes coding for gSNARE proteins were transcribed, we isolated total RNA from *Giardia* non-encysting trophozoites using TRIzol® reagent (Invitrogen) according to the manufacturer’s protocol. Samples then were treated with RNase-free DNase (Promega) to remove contaminating DNA. After one phenol-chloroform extraction, RNA was precipitated, and 5 μg was used to synthesize first-strand cDNA with SuperScript™ III reverse transcriptase (Invitrogen) and PolyT primer following the manufacturer’s instructions. One-tenth of the final material was used as a template for separate quantitative real-time PCR reactions with Quantitect™ SYBR® Green PCR (Qiagen) using the Chromo 4 real-time PCR detector. The same primers used in gSNARE antisense silencing were used to amplify fragments 100–500 pb in length. A negative control contained all reagents except the cDNA (RNA was included instead). One positive control was also used, the constitutively expressed gene *gdh* (GenBank™ accession number M84604) (58). We used Microsoft Excel® to open the exported Ct (threshold cycle) file. Reverse transcription PCR products were analyzed by electrophoresis on a 0.8% agarose gel in Tris acetate-EDTA buffer. Analysis of the DNA sequence was performed with the computer program DNASTar (Lasergene).

**Bioinformatic Analysis**—The latest version of the *Giardia* genome was downloaded. An ORF from the giardia13 assembly (ORF 10803) was included in the data set, as this ORF had been amplified by PCR in our laboratory and proved to be a SNARE protein through bioinformatics, although it was discontinued in the giardia14 assembly of the giardial genome. HMMsearch and HMMPfam were performed with the HMMER program (38). SMART searches were performed with the SMART Web server (39). HHPred (40) was performed with varying options, against either the Protein Data Bank or COG/KOG data bases. Secondary structure scoring was set to “predicted” versus “predicted only.” Searches that resulted in a top hit with a p value above 1e-4 were run through the HHSenser program (41). Phobius 5.1 was used to predict transmembrane domains and signal peptides (42).

Protein sequences were aligned with MUSCLE (43) and manually curated with Genedoc, taking into account secondary structure information. Accession numbers for proteins used in alignment were: ABA53519.1 (Syntaxin 1A), NP_001007026.1 (GOSR1), EAL24137.1 (Bet1), and CAG33270.1 (YKT6). The presence of Habc domains, characteristic of the syntaxin protein group, was determined using PCOILS (44), PSIPRED (45), and HHPred.

**RESULTS**

*G. lamblia* SNARE Homologs

Through runs of HMMsearch, HMMPfam (38), SMART (39), and HHPred (40) on the giardia13 genomic assembly, we identified 17 *Giardia* proteins as putative SNAREs (Table 1). The HMM profiles, recently published by Kloepper et al. (21) for several species, allowed us to classify these proteins more efficiently. Running HMMsearch with the HMM files described in Kloepper et al. (21) against the giardia14 assembly protein data base resulted in very few Qb and Qc proteins with E values smaller than the standard 0.1 E value cut-off line. Taking into consideration that *Giardia* is such a divergent eukaryote, whether because of primitiveness, rapid evolution, or a combination of both (5), we modified our protocol to increase the sensitivity of the search. All sequences with E values lower than 10 (285 protein sequences) were run through the HMMPfam program against a custom data base containing the complete Pfam-A data base and the HMMs (supplemental Table S1) (from Kloepper et al. (21)). All protein sequences in which the top hit was a non-SNARE HMM were removed from the data set.

To make a prediction about the possible function of the different SNAREs, we performed bioinformatic analyses expecting that functional conservation should be reflected by sequence conservation. Because the signals that drive the localization of SNAREs are not well understood, we searched for homologs of the entire sequence of each molecule and the SNARE domain only. FASTA searches against the Swiss-Prot and UniprotKB data bases provided homologs for each *Giardia* SNARE protein (supplemental Table S2). However, low E values prompted us to use a more sensitive searching method; thus HHPred and HHSenser were used to search the COG and KOG data bases (44) for the SNARE subfamily that is more closely homologous to each *Giardia* SNARE (supplemental Table S3).

Using HMMsearch and HMMPfam, we were able to classify putative SNAREs into four different groups (Qa, Qb, Qc, and R)
**SNAREs Define Vesicular Compartments in Giardia**

### TABLE 1

*Sequence characteristics of gSNARE proteins*

| Gene product | Function | ORF | GLS0803 | Length | SNARE domain | TM domain | Molecular mass | References |
|--------------|----------|-----|---------|--------|--------------|-----------|---------------|------------|
| Qa-SNARE 1   | Qa1      | 7,309 | 293     | 207–259 | 270–292      | 32.51     | 17, 19, 53    |
| Qa-SNARE 2   | Qa2      | 3,869 | 271     | 185–237 | 250–268      | 30.81     | 17, 19, 52, 53|
| Qa-SNARE 3   | Qa3      | 96,994| 307     | 217–269 | 284–305      | 34.79     | 53           |
| Qa-SNARE 4   | Qa4      | 11,270| 345     | 264–316 | 326–344      | 38.62     | 53           |
| Qa-SNARE 5   | Qa5      | 10,803| 268     | 185–237 | 248–265      | 30.46     | This study    |
| Qb-SNARE 1   | Qb1      | 16,054| 212     | 127–179 | 188–210      | 23.57     | This study    |
| Qb-SNARE 2   | Qb2      | 17,464| 204     | 124–176 | 182–201      | 22.45     | This study    |
| Qb-SNARE 3   | Qb3      | 5,161 | 215     | 122–174 | 184–204      | 24.54     | This study    |
| Qb-SNARE 4   | Qb4      | 5,785 | 226     | 125–173 | 186–206      | 24.74     | This study    |
| Qb-SNARE 5   | Qb5      | 10,315| 222     | 135–183 | 191–214      | 25.10     | This study    |
| Qc-SNARE 1   | Qc1      | 5,927 | 337     | 249–297 | 312–332      | 38.47     | This study    |
| Qc-SNARE 2   | Qc2      | 7,590 | 257     | 168–227 | 239–256      | 27.93     | This study    |
| Qc-SNARE 3   | Qc3      | 10,013| 220     | 128–176 | 189–207      | 24.46     | This study    |
| Qc-SNARE 4   | Qc4      | 19,509| 104     | 34–83   | 87–103       | 11.80     | This study    |
| R-SNARE 1    | R1       | 9,489 | 238     | 156–208 | 217–237      | 26.14     | This study    |
| R-SNARE 2    | R2       | 7,306 | 221     | 161–213 | CAAX         | 24.02     | This study    |
| R-SNARE 3    | R3       | 14,469| 239     | 152–204 | 70–90215–235 | 26.34     | This study    |

In accordance with genome-wide classification performed with other organisms. Five SNAREs corresponded to the Qa-SNARE subfamily (gQa-SNAREs 1–5), five to the Qb-SNARE subfamily (gQb-SNAREs 1–5), four to the Qc-SNARE subfamily (gQc-SNAREs 1–4), and three to the R-SNARE subfamily (gR-SNAREs 1–3) (Table 1). This classification was supported by performing local and global alignment HHPred searches against the Protein Data Bank data base, confirming that the top hit for each gSNARE corresponded to the Qa, Qb, Qc, or R SNAREs for the 2 nps Protein Data Bank entry (46) (supplemental Tables S4 and S5).

**Giardia SNARE Characteristics**

The putative gQ-SNAREs display all of the structural characteristics defined for this family of proteins. They have the typical size (104–345 amino acids), a single transmembrane domain near the C terminus (with a predicted type II integral membrane protein orientation), and a coiled-coil SNARE domain preceding the transmembrane domain (Table 1). In addition, using PCOILS (45), PSIPRED (47), and HHPfam, we could identify the presence of an N-terminal helical bundle (called Habc) (48) in gQa1, gQa2, and gQa3 (supplemental Fig. S1), characteristic of the syntaxin family, that can fold back into a closed conformation to interact with the SNARE motif (49). Secondary structure predictions for Qb-, Qc-, and R-SNAREs identified a similar series of helixes in gQb3, gQb4, gQb5, gQc1, and gQc3 (supplemental Figs. S2–S4). These could represent divergent Habc domains, as HMMPfam and HMMsearch were not able to detect homology to the Habc domain. These findings are consistent with the presence of Habc domains in members of the Qb and Qc families of *Leishmania major* and in human Syntaxin 6 (belonging to the Qc group) and Vti1 (belonging to the Qb group) (50).

Animals, plants, and fungi express SNAP-25 family proteins, which contain two SNARE motifs belonging to the Qb and Qc group. In addition, few Qb–SNAREs were reported to be present in protists like *Plasmodium falciparum* and *Dictyostelium discoideum* (21). *Giardia*, however, similar to other protozoa such as *L. major* and *Trypanosoma brucei* (51), does not possess any member of this SNARE family. All of the SNARE sequences identified contain a single, C-terminal SNARE motif.

As shown in Table 1, four gQ-SNAREs sequences belong to the specific syntaxin paralog family called gQa1–4, and these were characterized previously to this work (19, 32, 52, 53). We found that mAb 3B5 (raised against *Giardia* encysting trophozoite microsomes) recognizes gQa1 (supplemental Fig. S5). This mAb was useful in comparing the level of expression of the wild type gQa1 with the transgenic gQa1-HA expressed under the control of the α-tubulin promoter (supplemental Fig. S5). We also identified gQa2 by searching an incomplete version of the *Giardia* genome data base and subsequently performed sequencing of the entire gene from different cDNA and genomic DNA libraries (see additional methods and information in the supplemental data for Fig. S5). *Giardia* Qa3 and gQa4 were characterized similarly by other groups (52, 53).

The three gR-SNAREs correspond to the family of VAMP proteins and we called them gR1–3 (Table 1). Similar to findings in *L. major*, gR1–3 are longer than the classical short VAMPs (brevins) so they could be classified as long VAMPs (longins) (31). These three long gVAMPs contain the R-SNARE coiled-coil region. *Giardia* R1 and gR2 have the characteristic transmembrane domain near the C terminus; gR3 has an additional predicted transmembrane domain near the N terminus (amino acids 71–90). *Giardia* R2 has an isoprenylation site (CAAX box at amino acid 218) instead of the transmembrane domain, which mediates membrane attachment (Table 1).

**Giardia SNAREs Possess Atypical Amino Acid Residues at the “0” Layer**

It has been suggested that R-SNAREs provide an arginine residue (R) to the conserved ionic layer, whereas Q-SNARE provide complementary glutamines (Q) for the formation of the ternary complexes (26). As shown in Fig. 1, many putative *Giardia* SNAREs do not possess either residue at the zero layer. Present at this position were atypical amino acid residues that include aspartic acid (D), asparagine (N), serine (S), glycine (G), and isoleucine (I). Recently, atypical zero layer amino acids have been reported for other organisms, such as serine for the...
yeast Bet1p, aspartate for the human Vti1 and Slt1, and asparagine and isoleucine among members of the syntaxin subfamily of *P. falciparum* and in some synaptobrevins of *Plasmodium tetraurelia* (51). The presence of a glycine residue in place of glutamine at the zero layer position of gQc3 is a novel finding among the SNARE superfamily.

Subcellular Distribution of gSNARE Proteins in *G. lamblia*

Defining the subcellular localization of SNARE proteins is essential to understand their function, especially in an organism with an atypical secretory apparatus such as *Giardia*. To test whether the bioinformatic data correctly predict the compartment or pathway of a given SNARE, the 17 coding genes for putative gSNAREs were amplified by PCR from *G. lamblia* genomic DNA, cloned into an HA fusion vector, and transfected into *G. lamblia* trophozoites (37). It was shown previously that small tags such as the HA epitope do not influence the behavior of *Giardia* proteins and do not affect their subcellular localization (4, 20). The HA epitope was inserted at the N terminus of these constructs because SNARE proteins do not have an N-terminal signal peptide (31) and also because it has been shown that this procedure accurately reflects the localization of SNARE proteins in other eukaryotic cells (54). There was a possibility of mistargeting to improper locations because of overexpression, and thus care was taken to select cell clones expressing moderate or low levels of tagged proteins for localization experiments. We colocalized the fluorescence patterns obtained for each gSNARE with cellular markers to tentatively identify the labeled compartments. In addition, all SNAREs were analyzed under two developmental conditions, non-encysting as well as encysting cells. The latter displays a unique secretary compartment, secretory granules called ESVs, which seem to develop directly from the ER, as shown recently (55).

*Giardia* Qa-SNAREs—Immuno-fluorescence assays in non-encysting *Giardia* trophozoites showed that HA-gQa1 localized at the PVs, as determined by their colocalization with the mAb 5B11, which specifically labels PVs by recognizing lysosomal cathepsin B3 (Fig. 2A). The same pattern was observed using either the anti-HA mAb or the gQa1-specific mAb 3B5, showing that the HA tag does not affect the localization of this protein (supplemental Fig. S5). When these cells were induced to encyst, HA-gQa1 still localized to the PVs (Fig. 2B). In addition, using the anti-HA mAb and anti-cyst wall protein 2 (CWP2) mAb, partial colocalization between HA-gQa1 and some encystation-specific ESVs that transport CWPs was observed in many encysting cells (Fig. 2B). This result indicates a close interaction between PVs and ESVs, necessary for the release of secretory granule content before extracellular cyst wall formation, as suggested previously (17).

In non-encysting cells, HA-gQa2 localized to the ER surrounding both nuclei of the parasite (Fig. 2A), as determined by its colocalization with the ER-resident chaperone BiP (14). During encystation, HA-gQa2 showed the same ER pattern as well as colocalization with ESVs (Fig. 2B).

In non-encysting and encysting cells, HA-gQa3 appeared on broadly distributed punctate structures distributed throughout the cell cortex. Moreover, partial localization at the ER also was observed, particularly in the perinuclear region. On the other hand, the staining at the cell cortex colocalized with mAb 5B11, suggesting that HA-gQa3 also localizes to the PVs (Supplemental Fig. S6). In encysting trophozoites, gQa3 showed the same pattern, with less staining surrounding the nuclei, and the vesicles were in the proximity of, but distinct from the ESVs (Fig. 2B).

In non-encysting and encysting cells, HA-gQa4 localized in a punctate pattern at the ER and surrounded both nuclear enve-

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3 H. D. Lujan, unpublished results.
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FIGURE 2. Localization of gQA-SNAREs (gSyntaxins) in non-encysting and encysting Giardia trophozoites. A, confocal microscopy of direct immunofluorescence assays of non-encysting transfected Giardia trophozoites shows the colocalization (yellow) of HA-gQA1 (green) with the PVs (in red) and of HA-gQA2 and HA-gQA4 (green) with BiP (red) at the ER. HA-gQA3 localizes at internal vesicles, nuclear envelope, and PVs/plasma membrane (green); and HA-gQA5 (green) colocalizes (yellow) with the PM (VSP1267, in red). HA-tagged gQ-SNARE proteins are stained with FITC-conjugated anti-HA mAb (left panels); the PVs and BiP are detected with Texas Red-labeled (red) mAbs 5B11 and 9C9, respectively. B, confocal microscopy of direct immunofluorescence assays showing the localization of HA-gQA SNAREs (green) in encysting cells. The ESVs are labeled with mAb 7D2 specific for CWP2 (red).

lopes (Fig. 2). During encystation, HA-gQA4 displayed this pattern as well as colocalization with ESVs (Fig. 2B). In non-encysting trophozoites, HA-gQA5 was found not only at the PM colocalized with the integral membrane protein VSP1267, as determined using the specific mAb 5C1 against this protein (S6) (Fig. 2A), but in addition, it colocalized with PVs (supplemental Fig. S6), which indicates an interaction between PVs and PM, showing that the PV is a dynamic endosomal compartment. This interaction was confirmed by colocalizing the PVs (using mAb 5B11) with the PM (using mAb 5C1) in non-transfected trophozoites (supplemental Fig. S6). During encystation of these cells, we observed that the localization of HA-gQA5 remained at the PM/PVs interphase.

Thus, the gQA-SNAREs display distinct localizations: gQA1 to the PVs, gQA2 and gQA4 to the ER, gQA5 to the PM/PVs, and gQA2, gQA4, and gQA3 to membranes that are either subdomains of the ER or are closely associated with it. In addition, gQA4 appears to localize to ESVs during the process of encystation.

Giardia Qb-SNAREs—Immunofluorescence assays of transfected trophozoites reflected that both HA-gQB1 and HA-gQB2 localized in a similar vesicular pattern but one that had not been described before in non-encysting and encysting cells, e.g. lack of colocalization to the ER, PVs, or ESVs (Fig. 3). However, as some ER proteins display a dot-like staining pattern, it is possible that HA-gQB1 and HA-gQB2 localize to some portions of the ER or to ER exit sites (S5, S19).

In non-encysting trophozoites, HA-gQB3 was found in a number of vesicles with a distribution similar to "mitosomes" (S57), as a vesicular tube between the two Giardia nuclei and in close proximity to the basal bodies, and in vesicles at the lateral and posterior parts of the cell. These vesicles were clearly distinct from the ER and the PVs located underneath the PM and clearly similar to the mitosomes described in Fig. 2 of Dolezal et al. (S57). During encystation, the number of vesicles decreased and the pattern changed; lateral vesicles disappeared and a prominent vesicle appeared in the apical, posterior part of the cell, which conceivably is a result of the fusion of vesicles (Fig. 3B). However, the tubular vesicle found between the nuclei had the same localization pattern described by Dolezal et al. (S57).

In non-encysting and encysting cells, HA-gQB4 was found not only at the PM colocalizing with mAb anti-VSP1267 (Fig. 3) but also colocalizing with the PVs labeled with mAb 5B11 (supplementary Fig. S6).

HA-gQB5 localized at the ER in non-encysting cells as determined by its colocalization with BiP (Fig. 3A). When these cells were induced to encyst, HA-gQB5 still showed a more prominent perinuclear distribution with less staining at the ER. Interestingly, colocalization with ESVs also was observed near the nuclei (Fig. 3B).

Overall, the distribution of the gQB-SNAREs is somewhat different than that for the gQA subfamily. Giardia Qb4 and Qb5 can be assigned with high confidence to the PVs and the ER, respectively, but Qb1 and Qb2 are more broadly distributed, perhaps in transport vesicles, ER patches, or ER exit sites. The Qb3 protein displayed a unique distribution that might correspond to the mitosome, a recently discovered organelle that seems to be a remnant of mitochondria (S57).

Giardia Qc-SNAREs—Immunofluorescence assays in non-encysting and encysting trophozoites showed that HA-gQC1 and HA-gQC4 localized at the PVs as determined by their colocalization with mAb 5B11 (Fig. 4). In addition, we observed colocalization of these two SNAREs with the PM labeled with mAb 5C1 (supplemental Fig. S7). Surprisingly, in encysting trophozoites, HA-gQC4 colocalized with ESVs in proximity to the PVs/PM (Fig. 4B). In non-encysting and encysting trophozoites, HA-gQC2 localized at the PM (Fig. 4). Moreover, HA-gQC2 colocalized with PVs (supplemental Fig. S7). HA-gQC3 local-
ized at the ER in non-encysting cells as determined by its colocalization with BiP (Fig. 4A). In contrast, in encysting trophozoites, HA-gQc3 showed a pattern of vesicles next to the ESVs without colocalizing with them. Therefore, most of the gQc-SNAREs could be assigned to the PVs/PM compartment with the exception of gQc3 that seems to localize at the ER. Moreover, gQc4 appears to localize to ESVs during the process of encystation.

**Giardia VAMPs**—Immunofluorescence assays of transfected trophozoites showed that HA-gR1 localized at the ER in either non-encysting or encysting cells (Fig. 5). In addition, during encystation, HA-gR1 colocalized with ESVs (Fig. 5). HA-gR2 was found mainly in a region surrounding the nuclei of the parasite and in a diffuse intracellular cytoplasmic pattern, colocalizing in some regions with the ER in non-encysting trophozoites and encysting trophozoites (Fig. 5A). However, in encysting trophozoites, HA-gR2 colocalized partially with ESVs (Fig. 5B). HA-gR3 was found at the PM in non-encysting and encysting cells (Fig. 5). Therefore, the distribution of gR SNAREs is similar to that of the gQa-SNAREs, with gR1 and gR2 localized to the ER and surrounding both nuclei of the parasite and gR3 to the PM. Furthermore, gR1 and gR2 appear to localize to ESVs during encystation.

**Expression of SNAREs in G. lamblia**

To verify which of the genes encoding for the 17 gSNAREs were transcribed, we isolated total RNA from *Giardia* trophozoites and performed quantitative real-time PCR using gene-specific primers. As shown in Fig. 6, top, the expected size of cDNAs corresponding to each gSNARE was amplified, establishing the expression of all of these SNARE proteins in proliferating G. lamblia trophozoites. By this analysis, we also demonstrated that the gQa5 (ORF: 10803), absent in the latest *Giardia* DB release, giar-
dia14, is expressed in this parasite, suggesting not only the existence of this protein in the genome but also that it is expressed normally. As a positive control, the glutamate dehydrogenase (gdh) transcript was amplified (58). When we compared the threshold cycle values (Ct) of each amplicon with gdh (Fig. 6, bottom), we observed that all of the transcripts except gR3 have slightly lower expression levels than gdh. We also determined that the expression levels of gQa2, gQa4, gQa5, and gQc1 were slightly lower when compared with the other SNARE genes.

**Western Blot Assays of Giardia Putative SNAREs Revealed Slowly Migrating Bands That Might Be Attributed to SDS-resistant Complexes**

SNARE heterocomplexes consist of a parallel four-helix bundle of extraordinary stability derived from one R-SNARE and three Q-SNARE motifs (26). The four helices form a coiled-coil structure that in most cases is SDS-resistant at temperatures of ~80 °C but not at boiling temperature (59). SDS-resistant SNARE complexes often exist in various forms with different electrophoretic motilities that give rise to multiple anti-SNARE-immunoreactive bands on Western blots (59). To verify whether SDS-resistant complexes exist in *Giardia*, proteins were extracted in SDS-sample buffer without boiling, separated by SDS-PAGE, blotted and immunostained with an anti-HA mAb. With this antibody, we were able to detect one or more bands with molecular masses between ~60 to >200 kDa, consistent with complexes formed by the conserved coiled-coil domain of putative gSNAREs in both non-encysting and encysting trophozoites (Fig. 7). Faster migrating species that corresponded to the molecular weight of the monomeric form were detected in boiled samples (supplemental Fig. S8). It is known that high molecular weight (HMW) bands are not detected in samples that have been boiled prior to electrophoresis. However, in *Giardia*, when the samples were boiled, we found some peculiarities that had not been described in other eukaryotes (59). First, some of the putative gSNAREs (gQa1–4; gR1–3) presented almost the same bands obtained in unboiled samples,
suggesting that the majority of HMW bands are not heat-sensitive (supplemental Fig. S8). Second, other putative gSNAREs, such as Qa5, Qb4, Qc1, Qc2, and Qc4, showed not only the HMW bands but also a derivative of the lower band that represents the monomeric form (supplemental Fig. S8). On the other hand, gQb1–3 and gQc3 presented only the lower band, with
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TABLE 2

Summary of Giardia SNARE localization

Subcellular localization of Giardia SNAREs determined by IFA was compared with the predicted localization determined by FASTA, HMMPfam, and HHpred. To determine the closest orthologous gene for each Giardia SNARE protein, FASTA searches were performed with standard parameters on the UniProtKB/Swiss-Prot data base. The homology of the SNARE domain to the HMMs defined in Kloepper et al. (21) allowed us to predict the localization for each SNARE protein using HMMPfam. By using HHpred and HPhenser, the subcellular localization of the closest homologous KOG entry for each Giardia SNARE was analyzed. Subcellular localization was experimentally determined by IFA using organelle markers for each compartment. Presumed intracellular sites are as follows: NE (nuclear envelope), ER, GA (Golgi apparatus), TGN, Psvg, VC (vacuolar compartment), ESVs, PM (plasma membrane).

| gSNARE | Accession GL50803 | FASTA | HMMPfam | HHpred | IFA localization in non-encysting cells | IFA localization in encysting cells |
|--------|-------------------|--------|---------|--------|----------------------------------------|-----------------------------------|
| gQa1   | 7,309             | EC     | PM      | PM     | PM/ESVs                               | PM/ESVs                          |
| gQa2   | 3,869             | TGN    | TGN     | GA/EC  | NE/ER                                 | NE/ER/ESVs                       |
| gQa3   | 96,994            | PM     | PM      | PM     | small vesicles/PM/PM/ESVs             | small vesicles/PM/PM/ESVs        |
| gQa4   | 11,220            | ER     | ER      | ER     | NE/ER/vesicles                        | NE/ER/vesicles                   |
| gQa5   | 10,803            | VC     | PM      | PM     | Vesicles                              | Vesicles                         |
| gQb1   | 16,054            | EC     | TGN     | VESICLES | NE/ER/vesicles                        | NE/ER/vesicles                   |
| gQb2   | 17,464            | GA     | GA      | GA     | Vesicles                              | Vesicles                         |
| gQb3   | 5,161             | ER     | ER      | ER     | Mitosomes                             | Mitosomes                         |
| gQb4   | 5,785             | No hit | EC      | ER/EC  | Vesicles                              | Vesicles                         |
| gQb5   | 10,315            | EC     | TGN     | TGN    | NE/ER/vesicles                        | NE/ER/vesicles                   |
| gQc1   | 5,927             | PM     | EC      | EC     | Vesicles                              | Vesicles                         |
| gQc2   | 7,590             | VC     | ER      | EC     | Vesicles                              | Vesicles                         |
| gQc3   | 10,013            | PM     | EC      | PM     | Vesicles                              | Vesicles                         |
| gQc4   | 19,509            | GA/ER-GA | EC     | EC     | Vesicles                              | Vesicles                         |
| gR1    | 9,489             | GA     | GA      | GA     | Vesicles                              | Vesicles                         |
| gR2    | 7,306             | GA/ER-GA | GA     | GA/ER-GA | NE/ER cytoplasm                      | NE/ER/vesicles                   |
| gR3    | 14,469            | TGN/EC | EC      | EC     | PM/ESVs                               | PM/ESVs                          |

more intensity, which corresponded to the molecular weight of the monomeric protein (supplemental Fig. S8). In higher eukaryotes, it is known that a property described for native SNARE complexes and for complexes formed from reconstituting SNARE proteins is the temperature dependence of their SDS resistance (59). To test whether this was also true for SDS-resistant complexes in Giardia, we investigated the temperature sensitivity of the higher bands found with some putative SNAREs such as gQc3, gQa5, and gQb4. When Giardia proteins were incubated in SDS-containing sample buffer for 5 min at different temperatures, the amount of HMW bands decreased with increasing temperatures for gQc3 but not for gQa5 and gQb4 (supplemental Fig. S8). Moreover, some occasional additional bands lower than the monomeric molecular weight of each gSNARE appeared, which likely were degradation products. In most cases were unable to observe behavior of gSNAREs under SDS-PAGE similar to that reported in higher eukaryotes, and we conjectured that it might be due to the particular amino acids present at the zero layers of SNARE proteins.

In addition, when we used the gQa1-specific mAb 3B5 instead of the anti-HA mAb, the monomeric form of ~33 kDa as well as a ~115-kDa band, which may represent an SDS-resistant complex, were found (supplemental Fig. S5). Thus, it is possible that the failure of the anti-HA antibody to detect the 115-kDa high molecular mass gQa1 complexes (and most likely more complexes formed by the other gSNAREs) is attributable to a hidden HA epitope. In any case, the structural basis for these multiple forms of SNARE complexes remains unknown. Accordingly, the question of whether only one or several, all, or none of the SDS-resistant complexes represent fusion-competent SNARE structures remains unanswered.

Down-regulation of Some gSNAREs Affects Cell Viability

To determine the functionality of individual gSNAREs in regard to cell viability and their participation in the secretory pathway, Giardia clone WB/1267 was transfected with vectors carrying fragments of 150–400 bp of the antisense sequence of each gSNARE. Cells were selected with puromycin (33) and analyzed by quantitative real-time PCR to determine the level of silencing of the corresponding gSNARE gene (reduction of RNA expression varied from 70 to 60%; see supplemental Fig. S9). No viable clones were obtained for gQa1, gQa2, gQa3, gQa4, gQb3, gR1, gR2, and gR3, suggesting that these SNAREs are essential. However, for gQa5, gQb1, gQb2, gQb4, gQb5, and gQc1–4 we obtained viable clones in which, when cultured in growth medium, neither cell viability nor proliferation was affected when compared with either untransfected trophozoites or trophozoites transfected with an HA-tagged sense SNAREs. After 48 h in encystation medium, trophozoites expressing antisense SNAREs formed cysts at normal levels (results not shown).

Golgi SNARE Homologs in Giardia Fail to Establish the Presence of a Typical Golgi Apparatus

As we have shown, gQa2, gQb5, gR1, and gR2, which do not localize to a Golgi-like structure, have roles in vesicle transport during the differentiation of Giardia. However, homologs to these SNAREs were predicted by sequence comparisons to the Golgi and trans-Golgi network (TGN; see Table 2). HHpred analysis of gQa2 indicated that gSNAREs are highly similar to the Syntaxin 16 subfamily (see supplemental Table S4). Syntaxin 16 is a ubiquitous protein in eukaryotic cells that localizes at the Golgi apparatus and TGN and is involved in protein transport in this organelle (60). Moreover, the closest subfamily for gQb5 is Vti1, which comprises SEC20, Vti1a, and Vti1b (see supplemental Table S4). Vti1A is localized predominantly to the Golgi and the TGN area in higher eukaryotes.

Giardia SNARE-R1 was found at the ER/nuclear envelope in non-encysting cells. Bioinformatics analysis predicted that this protein localizes at the Golgi/ER-Golgi and is homologous to the Sec22 subfamily of SNAREs (see supplemental Table S4).
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Sec22b functions in both anterograde (61) and retrograde (62) trafficking between the ER and the Golgi.

The expression of *Giardia* SNARE-R2 was highly concentrated, surrounding both nuclei with a diffuse intracellular cytoplasmic pattern, colocalizing in some areas with the ER, and consistent with ER localization in non-encysting trophozoites. *Giardia* SNARE-R2 was predicted to localize to the Golgi and is highly similar to Ykt6 (see supplemental Table S3). Ykt6 is localized to both the cytoplasm and membranes. In mammalian cells, the majority of the membrane-bound pool of Ykt6 is associated with the Golgi; however a broader perinuclear distribution also has been observed (63). Consistent with its extensive cellular distribution, Ykt6 has been implicated in multiple transport steps in the secretory pathway, including ER-to-Golgi transport as part of a SNARE complex composed of GosR1, GosR2, and Stt5 and in early/recycling endosome-to-TGN transport as a part of a SNARE complex composed of Bet1L, GosR1, and Stt5 (63, 64). The localization of gR2 at the ER and the role of its close homologs suggest that it may be involved in ER homotypic and heterotypic fusion events. Furthermore, other Golgi SNARE homologs such as gQb1 and gQb2 showed a pattern of vesicles scattered throughout the cytoplasm; these vesicles did not colocalize with the ER, ECV, or PVS.

*Giardia* Qb1 was predicted to localize to TGN/endosomes; its closest homolog is Vti1 from *S. cerevisiae*, which is known to be involved in endosomal or vacuolar trafficking steps (see supplemental Table S3). Vti1p functionally interacts with Pep12p in trafficking from the Golgi to the pre-vacuolar compartment and with Sed5p in retrograde trafficking to the cis-Golgi (65).

*Giardia* Qb2 was predicted to localize to the Golgi. Its closest homolog is the Golgi SNARE 28 subfamily (Gos28p), of which a member is Gos1p of *S. cerevisiae* (see supplemental Table S3), which directly interacts with Sed5p, is localized primarily to the Golgi complex, and likely functions in ER-Golgi and/or intra-Golgi transport (66). In addition, the closest homolog to *Giardia* gQa3 is the yeast Sed5p, a molecule that participates in the transport between the ER and the Golgi complex and also is found in vesicular structures throughout the cytoplasm (67), suggesting that gQa3 could be involved in the same trafficking pathway of gQb1 and gQb2.

**DISCUSSION**

The protein trafficking pathway in eukaryotes takes a common route from the ER through the Golgi apparatus to the TGN, where exit and sorting take place (68). *Giardia* trophozoites possess an endomembranous organization that includes two nuclei, ER, PVs, constitutive transport vesicles (5), and the so-called mitosomes (57). *Giardia* lacks other organelles typical of higher eukaryotes such as classical mitochondria, peroxisomes, and compartments highly involved in intracellular protein trafficking and secretion, such as secretory granules and the localized stack of cisternae that is the hallmark of the Golgi apparatus (2). In an early report, we showed that when the proteins that will form the cyst wall (CWPs) concentrate within *de novo* generated-specific secretory granules (or ESVs), a perinuclear structure labeled with the Golgi-specific marker NBD-ceramide could be observed (7). However, in a later study we demonstrated that ESVs develop directly from the ER (55), raising doubts about the nature of that NBD-ceramide-labeled structure. Because secretory granules form at the TGN in higher eukaryotes, it is still an open question as to whether a Golgi complex is present in this ancient eukaryote.

In the present work, we first identified and characterized the complete set of SNAREs in *Giardia* to define the extent of the endomembranous system, including the presence or not of a typical Golgi. From the 17 gSNARE proteins that we characterized, five represent Qa-SNAREs, five Qb-SNAREs, four Qc-SNAREs, and three R-SNAREs. Compared with other unicellular eukaryotes such as *P. falciparum* (18 SNAREs), *L. major* (27 SNAREs), and *Entamoeba histolytica* (31 SNAREs) and organisms such as *Drosophila melanogaster* (20 SNAREs), *S. cerevisiae* (24 SNAREs), *Homo sapiens* (36 SNAREs), and *A. thaliana* (54 SNAREs), *Giardia* has a relatively small number of encoded SNAREs. This is consistent with the very elementary repertoire of membrane-bound organelles that can be identified in *Giardia* (Fig. 8). Our bioinformatic analysis complements that recently reported by Kloepper *et al.* (21), as we were able to identify two additional SNAREs and demonstrate that the predicted subcellular localization of many of them conflicts with our experimental results (see below). Additionally, we have shown here that one particular gSNARE is expressed and functional in *Giardia*, although it is not present in the latest version of the *Giardia* genome data base.

We analyzed the expression patterns and subcellular localization of the entire SNARE repertoire of *Giardia*. Our results indicate that these proteins are functional and distinctively localized to known subcellular compartments, suggesting a role in regulating membrane trafficking to and from these organelles. Interestingly, by antisense knockdown assays we found that most of the conserved *Giardia* SNAREs seem essential, whereas others are dispensable, particularly those that do have atypical amino acids in the zero layer. This suggests that some SNAREs have essential functions, whereas others might have redundant roles that can be compensated for by other SNARE isoforms (69). In addition, because many of the gSNAREs are essential for the viability of the parasite, they may present useful targets for the development of drugs against this important human pathogen.

Remarkably, gQb3 localizes to vesicles with a peculiar pattern, similar to the distribution attributed previously to mitosomes (see Fig. 2 in Dolezal *et al.* (57)). During encystation, the number of these gQb3-containing vesicles decreased and the pattern changed. Mitosomes are small, double membrane-bound mitochondrial remnants that retain some functions in the synthesis of iron-sulfur clusters (70). The possible presence of one SNARE in a mitochondrial remnant is intriguing, as no SNARE molecules have been reported to mediate mitochondrial fusion in more evolved cells except for a splice isoform of VAMP1 that contains a mitochondrial targeting signal (71).

Based on our bioinformatic and biological results, we reason that gQa2, gQa3, gQa4, gQb5, gQc3, gr1, and gr2 are likely to play roles in homotypic fusion events in the ER and in transport through the ER/nuclear envelope compartment (Fig. 8). We found that gQa2, gQa4, gQb5, and gr1 localized to the nuclear envelopes and to the ER. Because the ER and nuclear envelope are a continuous structure, most of these SNAREs appear to be...
localized in both. During encystation, however, some of these SNAREs colocalized with the ESVs in some particular spots of the nuclear envelope and ER. We suggested previously that a region surrounding both nuclei may be a compartment that represents a specialized ER cisterna where the biogenesis of the ESVs takes place (7). It is possible, therefore, that gQa2, gQa4, gQb5, and gR1 have a specific role in ESV biogenesis during differentiation of Giardia trophozoites, further supporting the idea that these specific secretory granules form from the ER in this protozoan.

Giardia possesses PVs located underneath the plasma membrane that function as endosomes and lysosomes and are therefore considered a primitive endosomal/lysosomal complex (8) (Fig. 8). The participation of PVs also has been described as influencing secretory granule discharge during cyst wall formation (13), and PVs act as secretory organelles that release cyst wall-disrupting enzymes during excystation (72). We were unable to demarcate SNARE localization between PVs and the plasma membrane by immunofluorescence assays using organelle markers, probably because of the heavy traffic between them during exocytosis, endocytosis, and membrane recycling. We found eight gSNAREs present in PVs/PM, three Qa-SNAREs, three Qc -SNAREs, one Qb-SNARE, and one R-SNARE. Giardia SNAREs Qa1, gQa3, and gQa5 are predicted to be located in the PM or endosomal compartments. The presence of three different gQa-SNAREs suggests that at least three distinct SNARE complexes are present in this region of the endomembrane system, perhaps one for exocytosis, one for endocytosis, and one for PV-PV fusion. The closest homologs for gQa1, gQa3, and gQa5 from other organisms are Syntaxin 12, Syntaxin 1A, and Pep12, respectively. Syntaxin 12 and Pep12 function in endosomal transport, whereas Syntaxin 1A is required for exocytosis. Based on these homologies, we suggest that gQa3 is essential for Giardia exocytosis, whereas gQa1 and gQa5 are important for endosomal trafficking.

Among the R-SNAREs, gR2 is most closely related in its SNARE domain to Ykt6. Ykt6 is unique in associating with membranes via a C-terminal isoprenylation and palmitoylation rather than through a transmembrane domain anchor. Interestingly, gR2 also contains a consensus sequence for isoprenylation at its C terminus as well as a second cysteine
nearly that could be palmitoylated. Mutation of both of the putative modified cysteines causes a redistribution of the protein from the membrane to the cytoplasm (supplemental Fig. S10). The conservation of lipidation of this SNARE suggests that a reversible membrane anchor may be important for the shuttling of the protein between different compartments.

gQb1, gQb2, and gQa3, which are most closely related to known Golgi SNAREs, were found in a punctate pattern that did not localize to any previously identified Giardia organelle, suggesting that these vesicles could be vestigial or remnant Golgi membranes. Organelles that resemble this pattern (e.g. punctate appearance scattered throughout the cytoplasm) are the “atypical” Golgi apparatus of the fungi Saccharomyces, Arp-ergillus, and Pisolithus (73, 74). Nevertheless, there are clear differences between Giardia and those organisms regarding glycosylation (one of the classical known functions of the Golgi apparatus). It was recently shown that, except for the first two steps that take place in the ER, complex N-glycosylation is missing in Giardia (75). When we performed studies evaluating glycosyltransferase activities during encystation (the process during which a carbohydrate-rich cyst wall is generated), we utilized a Giardia protein extract in the reaction. The enzymatic activities we measured at that time (7) may well correspond to the recently identified cyst wall synthase, a developmentally regulated enzyme that produces a cyst wall carbohydrate polymer (76). Therefore, these activities, which we suggested were present in Golgi-like compartment, apparently were unrelated to typical Golgi glycosyltransferases. Unfortunately, the lack of specific Golgi markers in Giardia prevented us from further characterization of these compartments. Additional evidence, however, plays against the possibility that vesicles containing gQb1, gQb2, or gQa3 are Golgi equivalents. For example, (a) when we expressed an HA-tagged version of a heterologous glycosyltransferase or p115 in trophozoites, they localized in the perinuclear region of Giardia and not in a punctate pattern dispersed throughout the cytoplasm (55); (b) a similar perinuclear pattern was observed by constitutive expression of the Giardia KDEL receptor (55); and (c) brefeldin A treatment of trophozoites expressing gQb1, gQb2, or gQa3 failed to disrupt these vesicular structures, whereas the secretory pathway was certainly affected, as determined by relocation of gARF3 (the only gARF showing similar localization pattern) (not shown). Although those experiments do not provide a definitive answer whether the vesicles containing SNAREs Qb1 and Qb2 are Golgi structures, our results strongly suggest that if Giardia has a Golgi, it is completely atypical.

In summary, we have added new evidence regarding the lack of a typical Golgi apparatus in this ancient eukaryote. However, some features of the Golgi, such as the packaging and sorting function, seem to be performed by the ER or the nuclear envelope, raising the question of whether the typical architecture of the Golgi in higher eukaryotes is a cause or consequence of the presence of glycosyltransferases needed for the biosynthesis of complex glycoconjugates, molecules that are missing in Giardia (2, 75).

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