Glucosylceramide Transferase Activity Is Critical for Encystation and Viable Cyst Production by an Intestinal Protozoan, Giardia lamblia*

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Background: The production of viable cysts by Giardia is essential for transmitting the infection via contaminated food and water.

Results: Overexpression and knockdown of glucosylceramide transferase activity affect encystation, cyst viability, and overall lipid balance in Giardia.

Conclusion: Regulated expression of glucosylceramide transferase is linked to encystation and cyst production.

Significance: Glucosylceramide synthesis could be targeted for developing novel anti-giardial therapy.

The production of viable cysts by Giardia is essential for its survival in the environment and for spreading the infection via contaminated food and water. The hallmark of cyst production (also known as encystation) is the biogenesis of encystation-specific vesicles (ESVs) that transport cyst wall proteins to the plasma membrane of the trophozoite before laying down the protective cyst wall. However, the molecules that regulate ESV biogenesis and maintain cyst viability have never before been identified. Here, we report that giardial glucosylceramide transferase-1 (gGlcT1), an enzyme of sphingolipid biosynthesis, plays a key role in ESV biogenesis and maintaining cyst viability. We find that overexpression of this enzyme induced the formation of aggregated/enlarged ESVs and generated clustered cysts with reduced viability. The silencing of gGlcT1 synthesis by antisense morpholino oligonucleotide abolished ESV production and generated mostly nonviable cysts. Interestingly, when gGlcT1-overexpressed Giardia was transfected with anti-gGlcT1 morpholino, the enzyme activity, vesicle biogenesis, and cyst viability returned to normal, suggesting that the regulated expression of gGlcT1 is important for encystation and viable cyst production. Furthermore, the overexpression of gGlcT1 increased the influx of membrane lipids and fatty acids without altering the fluidity of plasma membranes, indicating that the expression of gGlcT1 activity is linked to lipid internalization and maintaining the overall lipid balance in this parasite. Taken together, our results suggest that gGlcT1 is a key player of ESV biogenesis and cyst viability and therefore could be targeted for developing new anti-giardial therapies.

Giardiasis, caused by Giardia lamblia, is widespread throughout the world. This intestinal parasite exists in two morphologic forms, trophozoites and cysts. Although trophozoites colonize the small intestine and produce infections, cysts transmit the disease via contaminated water and food (1). Stage-specific differentiation of cyst to trophozoite, which takes place in the human stomach, is known as encystation, whereas the transformation from trophozoites to cysts that occurs in the small intestine is called encystation. Giardial cyst walls contain insoluble filamentous materials made of proteins, glycoproteins, and polysaccharides (2–5). At the onset of encystation, encystation-specific vesicles (ESVs)3 are synthesized by trophozoites that transport three cyst wall proteins (CWP-1, -2, and -3) to the plasma membrane before laying down the water-resistant cyst wall (5). CWP-1, -2, and -3 are acidic proteins (molecular mass ~26, ~39, and ~27 kDa, respectively) containing similar domains, including N-terminal signal sequences and five tandem, leucine-rich repeats (6, 7). Reports suggest that all three CWPs are essential for forming ESVs and that CWP-2 functions as an aggregation factor by interacting with CWP-1 and CWP-3 via conserved regions (8). Besides these three CWPs, high cysteine, nonvariant cyst protein, and cysteine protease-2 were also shown to participate in the process of encystation and cyst production (5, 9).

3 The abbreviations used are: ESV, encystation-specific vesicle; CWP, cyst wall protein; GlcCer, glucosylceramide, GlcT1, glucosylceramide transferase 1; gGlcT1, giardial glucosylceramide transferase-1; GCS, glucosylceramide synthase; SL, sphingolipid, GM1, mono-sialotetrahexosylceramide; GM3, mono-sialodihexosylceramide; GD3, di-sialodihexosylceramide; SP2, serine palmitoyltransferase; PMP, α-threo-1-phenyl-2-palmitoylamino-3-morpholin-1-propanol; PDMP, α-threo-1-phenyl-2-decanoylamino-3-morpholin-1-propanol; NBD, N-[7-(4-nitrobenzo-2-oxa-1,3-diazole)]-Bodipy, 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene; PC, phosphatidylcholine; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; SM, sphingomyelin; FDA, fluorescein diacetate; PI, propidium iodide; FAST Dil, 1,1'-dilinoleyl-3,3,3',3'-tetramethylindocarbocyanine; ESI, electrospray ionization; GSL, glycosphingolipid; ER, endoplasmic reticulum.

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Giardial GlcT1 Activity and Cyst Viability

Emerging reports indicate that the enzymes and products of sphingolipid (SL) biosynthesis pathways act as regulators of various cellular processes in both lower and higher eukaryotes. For instance, ceramide synthase activity is associated with increased tumor growth and progression in head and neck cancers (10). Sphingosine 1-phosphate has been shown to generate initial signals for apoptotic cell extrusion (11). Ceramidase expression is important for normal retinal function, and the targeted expression of neutral ceramidase in mutant fruit flies rescues photoreceptor degeneration (12). The mutation in the long chain sphingoid base subunit-1 (LCB1) of serine palmitoyltransferase causes autosomal, dominant, peripheral, and sensory neuropathy (13). In plants, the synthesis of four different classes of SLs and glycosphingolipids (GSLs) was observed, including glycosylinositolphosphoceramides, glycosylceramides, ceramides, and free LCBs. Several enzymes of plant SL metabolic pathways have also been cloned and characterized (14). SLs are also critical for regulating various biological processes in unicellular organisms like fungi and protozoa. In budding yeast, SLs contain C26 acyl moieties and are connected during host-parasite interactions (16).

For instance, ceramide synthase activity is associated with increasing the influx of lipids, cholesterol, and fatty acids from the culture medium.

**EXPERIMENTAL PROCEDURES**

**Materials**—Unless otherwise specified, all chemicals were purchased from Sigma and were of the highest purity available. Tetramethylrhodamine (TMR)-conjugated goat anti-mouse antibodies, fluorescent (Bodipy- or NBD-conjugated) lipids, 1,1’-dilinoleyl-3,3,3’,3’-tetramethylindocarboxyamine (FAST Dil), and 4’,6-diamidino-2-phenylindole (DAPI) were purchased from Invitrogen. PPMP and other lipid standards, including $^{13}$C-deuterated GM3, were purchased from Matreya LLC (Pleasant Gap, PA). Glucosylceramide and lactosylceramide were obtained from Avanti Polar Lipids (Alabaster, AL). Anti-giardial cyst and anti-AU1 antibodies (monoclonal) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and Covance Laboratory (San Diego, CA), respectively. UDP-[$^{14}$C]glucose (300 mCi/mmol) was purchased from American Radiolabeled Chemical (St. Louis, MO).

**Trophozoites, Encysting Cells, and the Generation of in Vitro Cysts—**G. lambia trophozoites (strain WB, ATCC No. 30957) were cultivated following the method of Diamond et al. (19) using modified TYI-S-33 medium supplemented with 5% adult bovine serum and 1% bovine bile (20). The antibiotic pipercillin (100 μg/ml) was added during routine culturing of the parasite (21). Trophozoites were detached from the culture flask by ice chilling and harvested by centrifugation at 1500 × g for 10 min at 4°C, followed by three washings in sterile PBS and microscopic determination of cell numbers using a hemocytometer. In vitro encystation was carried out by culturing the trophozoites in TYI-S-33 medium, pH 7.8, supplemented with adult bovine serum (10%, v/v), lactic acid (5 mM), and porcine bile (250 mg/ml) for various time points, as described below (21). Cells were allowed to encyst for 72 h, and cysts were isolated by centrifugation (2,500 × g for 10 min at 4°C), washed three times in cold distilled water, and kept in water for 3 days in a refrigerator (4–8°C). Isolated water-resistant cysts were counted or subjected to the microscopic experiments also described below.

**Overexpression of gGlcT1 in Giardia Trophozoites—**For overexpression, a small peptide epitope (AU1)-tagged pNT5 expression plasmid (obtained from Dr. Chin-Hung Sun, Taiwan) containing the gglct1 gene was constructed following the method described by Pan et al. (22). Briefly, the entire open reading frame of gglct1 ORF_11642 was amplified by PCR using the primers 5’-GCAGCCATGATGAGGGTTGACTCTC-TCC-3’ and 5’-GGGAGATTCTCAGTATCGATACGT-ATCGTCAGGGATTTTTT-3’. The insert was digested with EcoRI/NcoI and ligated into EcoRI/NcoI-digested dephosphorylated pNT5 plasmid (22). This new plasmid pNT5-gglct1 was then transformed into competent DH5α cells and plated onto LB plates containing 100 μg/ml ampicillin. Colonies were screened by PCR, and positive colonies were sequenced at the University of Texas–El Paso DNA core facility. The ABI Prism BigDye Terminator version 3.1 cycling sequencing kit (Applied Biosystems, Carlsbad, CA) was used to amplify the DNA with fluorescently labeled dideoxynucleotides. The sequencing reaction was subsequently cleaned using Agencourt Clean SEQ (Beckman Coulter, Brea, CA). The parasites were placed in a 4-mm electroporation tube (Fisher Biotech, Waltham, MA) in a 300–μl suspension of TYI-SS media; 40 μg of the pNT5-gglct1 plasmid was resuspended in the cell suspension. Trophozoites were then transfected by electroporation on a Gene Pulser X cell (Bio-Rad) using the following parameters: 322 V, 500 μF, and 1.5 mmol of the pNT5-gglct1 plasmid was resuspended in the cell suspension. Trophozoites were then transfected by electroporation on a Gene Pulser X cell (Bio-Rad) using the following parameters: 322 V, 500 μF, and 1.5 mmol of the pNT5-gglct1 plasmid was resuspended in the cell suspension. 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followed by selection with 100 μg/ml G418 (Sigma). Stable transfectant trophozoites overexpressing gGlcT1 (+gGlcT1) were established within 2 weeks.

**Knockdown of gGlcT1 Expression by Antisense Morpholino Oligonucleotide**—Cultured *Giardia* trophozoites were harvested by centrifugation as described above. Parasites were placed in a 4-mm electroporation tube (Fisher Biotech) in a 300-μl suspension of the media, transfected (using the same setting as the overexpression described above) with anti-ggclt1 (5’-CTAAGGAGAGGTCAACCCGTCAT-3’), and scrambled (mismatch) with (5’-CCTTACCTCAGTACATTATA-3’) morpholino oligonucleotides (custom ordered from Gene Tools, Inc., Philomath, OR) following the protocol described previously by Carpenter and Cande (23). gglc1-trophenotozoites were left to recover for 6 h for additional experimentation.

Assessing PPMP and Morpholino Oligonucleotide-treated Trophozoites—Giardial trophozoites were electroplated with 50 μM of anti-ggclt1 morpholino and/or treated with PPMP (10 μM) for 24 h. Cells were then harvested and mounted with Prolong® Gold antifade reagent (Invitrogen) in a four-well chamber slide as described above without any further treatments. At least 100 singlet, doublet, and triplet trophozoites from 10 different fields were viewed and counted by confocal microscopy (Zeiss-LSM 700) using Zen 2009 software (Carl Zeiss, Irvine, CA). The percentage values of doublet cells were calculated based on the total number of cells (singlet, doublet, and triplet).

**Rescue Experiments**—The stable transfectants of *Giardia* trophozoites overexpressing gGlcT1 were generated as described above. gGlcT1-overexpressing trophozoites were then transfected with anti-ggclt1 morpholino oligonucleotide following the procedure described above. After antisense morpholino oligonucleotide transfection, gGlcT1-overexpressing trophozoites were allowed to recover for 6 h before conducting the experiments described below.

**gGlcT1 Assay**—The activity of the gGlcT1 enzyme in *Giardia* trophozoites, encysting cells, cysts, gGlcT1-overexpressing, and gGlcT1-knockdown cells was measured using the methods described for mammalian and *Plasmodium* cells (24, 25). Each reaction tube contained phosphate buffer (100 mM, pH 7.4), enzyme extracts (1500 x g supernatant), UDP-[14C]glucose (~100,000 cpm/assay), protease inhibitors (2.5% E-641, 1 mM MgCl2, 2 mM KCl, 2 mM β-NAD, and the liposomal substrate containing 0.1 nmol of ceramide mixed with dipalmitylphosphatidylcholine in a ratio of 1:10 (v/v) (total volume, 100 μl). The mixture was incubated for 5 h at 37°C and extracted with chloroform (CHCl3)/methanol (MeOH), 1:1 v/v, and the formation of enzymatic product ([14C]GlcCer) was assessed by measuring the radioactivity in a scintillation counter (Beckman, Brea, CA). The assay was conducted in the presence and absence of PPMP (10 μM), a common inhibitor of GlcCer synthesis (26).

**Synthesis of NBD-C6-Glucosylceramide**—NBD-GlcCer synthesis by *Giardia* was monitored following the method described by Gupta et al. (27). Briefly, cells were harvested and resuspended in PBS supplemented with 1% glucose and 0.2% L-cysteine. A total of 50 μM of NBD-C6 ceramide-BSA complex (Invitrogen) was added and incubated for 5 h at 37°C. Sphingolipids were extracted in a solvent in a mixture containing 1.3 ml of acetic acid/MeOH (1:50, v/v), 1.3 ml of CHCl3, and 1.3 ml of Milli-Q H2O (28). The lower CHCl3 phase was pooled and dried under nitrogen. Samples were resolubled on TLC (Whatman) using the following solution: 170 ml of CHCl3, 30 ml of MeOH, and 2 ml of 3.5% ammonium hydroxide (NH4OH). The formation of NBD-C6-glucosylceramide was visualized under a UV lamp (27).

**Staining with Anti-cyst Antibody**—To evaluate the transition from nonencysting to encysting trophozoites and cysts as well as to identify ESVs, cells were labeled with anti-cyst antibody (1:100) and analyzed by confocal microscopy. This anti-cyst antibody purchased from Santa Cruz Biotechnology was raised against intact cysts and therefore recognizes all cyst wall proteins (CWP1, CWP2, and CWP3). Trophozoites were cultured and subjected to encystation in a cultured medium as described before (21). Cells were harvested, transferred, and fixed to four-chambered slides using 4% paraformaldehyde. Fixed samples were allowed to react with anti-cyst antibody overnight at 6–10°C, followed by reacting with tetramethylrhodamine-conjugated goat anti-mouse antibody (1:500) (Invitrogen) for 2 h at room temperature. Cells were subsequently mounted with Prolong® Gold antifade reagent mixed with DAPI (Invitrogen). Samples were analyzed in a confocal microscope (Carl Zeiss Laser Scanning Systems LSM 700), using the Zen 2009 software (Carl Zeiss) for acquisition and image analysis.

**Labeling Trophozoites with Fluorescent-conjugated Lipid Probes**—Control and gGlcT1-overexpressing trophozoites were harvested and allowed to adhere in the Nunc Lab-Tek II 4-well chamber slide (Thermo Fisher Scientific, Inc., Austin, TX) for 30 min at 37°C. The cells were washed with PBS and incubated with different fluorescent lipid probes, i.e., Bodipy-phosphatidylcholine (Bodipy-PC; 100 nM); NBD-phosphatidylglycerol (NBD-PG, 1 μM); Bodipy-phosphatidylethanolamine (Bodipy-PE, 100 nM); Bodipy-ceramide (200 nM, Bodipy-sphingomyelin (Bodipy-SM, 200 nM); or FAST Dil (1 μM) for 1 h at 37°C as described previously (28). The cells were then fixed with 4% paraformaldehyde, washed with PBS, mounted with ProLong® Gold Antifade Reagent containing DAPI (Invitrogen), and visualized by confocal microscopy (Carl Zeiss Laser Scanning Systems LSM 700). The fluorescence intensities were quantified with the help of the Zeiss ZEN 2009 confocal software (Carl Zeiss).

**Monitoring Cyst Viability**—Fluorescein diacetate (FDA) inclusion and propidium iodide (PI) exclusion experiments were performed to monitor cyst viability. Briefly, a 25 mM stock solution of FDA and PI (Sigma) was prepared in 100 mM phosphate buffer, pH 6.0, from which a working solution was made by adding 40 μl of stock to 10 ml of phosphate buffer. gGlcT1-overexpressing and gGlcT1-knockdown trophozoites were subjected to encystation as described above, and the water-resistant cysts were collected by centrifugation. Approximately 107 cysts were subjected to FDA and PI staining as described previously by Gillin et al. (21). Cysts were then washed with PBS, mounted on a slide with DAKO mounting media (DAKO,
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Carpentia, CA), and viewed under the Zeiss LSM 700 confocal microscope using Zen 2009 software (Carl Zeiss).

Immunoblotting Analysis—Giardial cells were isolated and resuspended in lysis buffer (5 mM Tris-HCl, 2 mM EDTA, 0.1% Triton X-100, pH 7.4) containing protease inhibitor mixture and E-64 (1 μM, Sigma). The sample was freeze thawed at −80 °C, boiled in sample buffer before being analyzed (40 μg protein/lane) by 10% SDS-PAGE, and followed by immunoblotting analysis on the polyvinylidene fluoride (PVDF) membrane. The membrane was blocked in 1% BSA, incubated with anti-cyst antibody (monoclonal, 1:100), and incubated overnight at 4 °C. The PVDF membrane was then incubated with anti-mouse HRP conjugate (1:10,000; KPL, Inc., Washington, D.C.) for 30 min at room temperature. The enhanced chemiluminescence (ECL) technique was used for the detection of protein bands.

Sterol Analysis by GC-MS—Sterol analysis in control and gGlcT1-overexpressing trophozoites was carried out according to the method of Fridberg et al. (29). Trophozoites (~1 × 10⁸) were grown and harvested as described above, resuspended in CHCl₃/MeOH/H₂O (1:2:0.8, v/v/v), centrifuged (1500 × g) at room temperature for 15 min. The supernatant was collected and dried under nitrogen, and the pellet was then extracted with CHCl₃/MeOH (2:1, v/v) three times and with CHCl₃/MeOH: H₂O (1:2:0.8, v/v/v) twice before being dried under a nitrogen stream. The supernatants obtained from these six extractions were pooled together before being dried in the same flask. The dried samples were then submitted to Folch’s partition, after first being diluted in CHCl₃/MeOH/H₂O (4:2:1.5, v/v/v), and centrifuged (1500 × g) at room temperature for 15 min. This partitioning gave rise to two phases as follows: a lower phase, rich in free fatty acids, sterols, and phospholipids, and an upper phase, rich in inositol phospholipids. The lower phase was dried down with nitrogen gas, resuspended in pure CHCl₃, and fractionated through a Silica Gel 60 column according to Pernet et al. (30). The CHCl₃ phase of the samples were dried under a nitrogen stream and dissolved in dichloromethane. Fifty ng of external standard (Stigmasterol, Sigma) was added to the giardial sample, and 1 μl of the resulting mixture was injected into the gas chromatographer (Trace GC, Thermo Fisher Scientific) coupled to a mass spectrometer (Polaris Q, Thermo Fisher Scientific) (GC-MS). The fractionation was performed in a TR-5 ms column (30 m × 250 mm × 0.25 μm, Thermo Fisher Scientific). The injector temperature was set at 250 °C. The gradient was set at an initial temperature of 170 °C for 3 min, followed by a 20 °C/min increase up to 280 °C, and then a 17-min hold after the final temperature was achieved. The carrier gas was helium, with a flow rate of 1.2 ml/min. The samples were ionized by electron impact (EI) at 70 eV and 30 °C, and the spectra were collected at the 50–650 m/z range.

Fatty Acid Methylation and GC-MS Analysis—Fatty acids extracted from Giardia were subjected to methylation following the protocol described by Maldonado et al. (31). Briefly, a methanol fraction of lipid extracts from trophozoites (~1 × 10⁸) was dried under nitrogen, resuspended in 13 N NH₄OH/H₂O (1:1, v/v), and incubated for 1 h at 37 °C. The sample was then dried under a nitrogen stream, resuspended in MeOH, dried again, and mixed with 0.5 N methanolic HCl. The mixture was incubated for 1 h at 75 °C and then mixed with 0.5 N NaOH to neutralize the reaction before mixing with 750 μl of dichloromethane and H₂O (1:1, v/v). This mixture was then centrifuged at 1500 × g for 15 min, and the lower phase was separated. Standards were dissolved in dichloromethane and analyzed by GC-MS (Trace GC/Polaris Q, Thermo Fisher Scientific). The injector was set at 200 °C, and the following gradient was used: 70 °C for 5 min, followed by 4 °C/min up to 140 °C, 2 °C/min up to 185 °C, and 185 °C for 10 min. Helium was used as the carrier gas, with a flow rate of 1 ml/min, and then the molecules were ionized by electron impact at 70 eV and 200 °C. The spectra were collected at the 30–400 m/z range, and fatty acid species were identified by comparison with the FAME 37-methylated fatty acid mix standard (Supelco, Sigma).

Glycosphingolipid Analysis by ESI-MS-MS—Neutral and acidic glycosphingolipids (GSLs) were extracted and separated following the method described by Li et al. (32). Briefly, control, gGlcT1-overexpressed, gGlcT1-knockdown, and gGlcT1-rescued trophozoites (~1 × 10⁶ cells/sample) were harvested, pelleted, and extracted first with CHCl₃/MeOH (1:1, v/v) and then with isopropanol alcohol/hexane/H₂O (55:25:20, v/v/v) before being subjected to DEAE-Sephadex chromatography. Neutral lipids were eluted with CHCl₃/MeOH/H₂O (30:60:8, v/v/v). Acidic lipids were eluted with MeOH-containing 0.8 M sodium acetate. Both neutral and acidic GSLs were subjected to methylation as described by Ciucanu and Kerek (33), followed by ESI-MS/MS analysis using an LTQ XL mass spectrometer (Thermo Fisher Scientific). Briefly, samples were mixed with 1 pmol/μl methylated standards (d18:1/C12:0) and introduced via a TriVersa NanoMate nanoelectrospray source (Advion, Ithaca, NY) (capillary temperature 230 °C, with injection time of 100.00 ms, activation time of 30 ms, activation Q value of 0.250, isolation width of m/z 2.0, and acquisition time of 0.5 min). Quantitation was achieved using select reaction monitoring with collision energy set to 50 for all samples. For hexosylceramide reactions, singly charged parent ions with sodium adduct ([M + Na⁺]⁺) were fragmented and monitored for the fragment ions of permethylated hexose [C₁₆H₂₉O₆ + Na⁺]⁺ (from monohexosylceramide), permethylated dihexose [C₁₇H₃₀O₁₁ + Na⁺]⁺ (from dihexosylceramide), and permethylated trihexose [C₂₈H₄₂O₁₆ + Na⁺]⁺ (from trihexosylceramide). For the acidic GSLs (i.e. GM1, GM3, and GD3), the loss of a methylated sialic acid fragment at 376 Da was monitored at m/z 1266. For hexosylceramide was normalized to the internal standard d18:1/C12:0-glucosylceramide (Avanti Polar Lipids), and dihexosylceramide and trihexosylceramide were normalized to the internal standard d18:1/C12:0-lactosylceramide (Avanti Polar Lipids). GM3, GD3, and GM1 were normalized to ¹³C-deuterated d18:1/C18:0-GM3 standard (obtained from Matreya). Because of differences in ionization efficiencies between individual lipids, relative abundance was calculated rather than absolute mass of each lipid class.

Membrane Fluidity Assay—Approximately 10⁷ trophozoites (control and gGlcT1-overexpressing) were grown and harvested as described above, and the cells were analyzed using the
membrane fluidity kit (M0271, Marker Gene Technologies, Inc., Eugene, OR). Cold-shock trophozoites (4 °C, 30 min) were used as positive control. The cells were resuspended in 200 μl of perfusion buffer with 20 μM fluorescent lipid reagent (pyrene decanoic acid) and 0.08% of pluronic F127, provided with the kit. The suspension was shaken on a rocker for 1 h at RT, and the cells were then washed with PBS and subsequently analyzed using the ISS K2 Multifrequency Phase and Modulation Fluorometer (ISS, Inc., Champaign, IL). The samples were excited at 341 nm, and the emission spectra were recorded at 360–500 nm. Crossed polarizers on excitation and emission beams were used to reduce the background because of scatter. The emission spectra were then appended with normalized intensities using VINCI software (ISS).

**Statistical Analysis**—All values were given as mean values ± S.D., and statistical analyses were generated using SAS software version 9.2 (SAS Institute, Cary, NC) and Graphpad Quickcalcs. In experiments with three or more variables, the F-test was performed, and in experiments with two variables the Student’s t test was performed. Statistical values less than 0.01 were considered highly significant, whereas values less than 0.05 were considered significant. The variances of MS data for GSLs were calculated by one-way analysis of variance.

**RESULTS**

**gGlcT1 Activity Is Elevated during Encystation**

Earlier reports from this laboratory and other laboratories have indicated that GlcCer, a precursor of wide varieties of GSLs, play an important role during encystation by *Giardia* (17, 18). It was observed that the transcript of the gGlcT1 gene (*gglct1*) is up-regulated in encysting cells and that PPMP, an inhibitor of GlcCer synthesis (34, 35), inhibits the cyst production in culture (17). In this study, we asked if *Giardia* synthesizes the active gGlcT1 enzyme and uses it to drive the process of encystation. Therefore, gGlcT1 activity was measured in the cell-free extracts (1500 g supernatant) of nonencysting and encysting trophozoites as well as in water-resistant cysts using UDP-[14C]glucose and ceramide (24, 25). Results show that the basal gGlcT1 activity is low in nonencysting trophozoites and up-regulated in 12- and 24-h encysting cells and increases several-fold in cysts (Fig. 1A). We found that the gGlcT1 activities in trophozoites and encysting cells were inhibited by PPMP, which that bears a resemblance to both the ceramide and GlcCer (34, 35). However, unlike trophozoites and encysting cells, the cyst enzyme was completely resistant to PPMP (Fig. 1A). Next, we generated GlcT1 knockdown *Giardia* where the *glt1* gene was silenced by morpholine oligonucleotide. Knockdown by morpholine analogs is a faster and more efficient way to silence genes in *Giardia* (23). In recent years, various laboratories have successfully used antisense morpholine oligonucleotides to knock down giardial kinesin and flagellar genes. The effects of antisense morpholinos are transient and last for 72 h, which is sufficient to carry out one round of the encystation cycle (36). As shown in Fig. 1B, knockdown by anti-gGlcT1 morpholino reduced the gGlcT1 activity in cysts by ~60%. This result suggests that although resistant to PPMP, cyst gGlcT1 could be inhibited by anti-gGlcT1 morpholino, which blocks the translation initiation of gGlcT1 by targeting the 5’ region of the *glt1* gene. However, PPMP competes with ceramide for the catalytic site of gGlcT1 and is thereby expected to inhibit the enzymatic reaction (34).

Synthesis of GlcCer, the reaction product of gGlcT1, was also monitored by assessing NBD-GlcCer synthesis by encysting cells. For this, both nonencysting and encysting *Giardia* trophozoites were labeled with NBD-ceramide as substrates. The reaction was carried out for 5 h at 37 °C in the presence and absence of PPMP (10 μM). [14C]-Labeled GlcCer was extracted following the protocol described under “Experimental Procedures.” Data represent means ± S.D. of three separate experiments, and the experiments were carried out in duplicate (**, p < 0.01). 1C shows that the synthesis of NBD-GlcCer is extremely low (almost nonvisible in the photograph) in trophozoites and increases during encystation. However, we found that the formation of NBD-GlcCer in 12-h encysting cells is slightly higher than in 24-h encysting cells, which could be due to the fact that cells at the later stage of encystation (i.e. 18 h onwards) start forming cyst walls and become increasingly impermeable to NBD-ceramide and other molecules (data not shown). Therefore, in this study, no attempt was made to label the cyst with NBD-GlcCer. The increased synthesis of NBD-GlcCer during 12- and 24-h encysting *Giardia* supports our enzymatic results (Fig. 1A) that...
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Encystment stimuli induce gGlcT1 activity, leading to the synthesis of GlcCer.

Modulation of gGlcT1 Activity Affects ESV Biogenesis

*Giardia* has a relatively simple life cycle, i.e. replicative trophozoites and relatively dormant cysts. During encystment in the small intestine, trophozoites synthesize ESVs that are necessary to transport CWPs to plasma membranes (37). ESVs are synthesized in the endoplasmic reticulum (ER), located in the perinuclear membranes/cytoplasm in early encysting cells, and then distributed extensively in the periphery of the cell (in close proximity to peripheral vacuoles) in the late encystation phase (38). Because of the presence of Golgi-like cisternae in ESVs, these sorting vesicles are also considered as a primitive Golgi complex of *Giardia* (39). Over the years, many investigators have studied encystation and identified various proteins and genes that are associated with the process (see Ref. 5 for review). However, nothing is known about how the ESV biosynthesis is regulated and participates in viable and infective cyst formation. Because gGlcT1 activity is up-regulated during encystation (Fig. 1 A), we had thought that gGlcT1 was linked to ESV biogenesis. To test this, control and gGlcT1-knockdown trophozoites were subjected to encystation for 18 h, and ESV synthesis was monitored using an anti-cyst antibody that recognizes early and late ESVs as well as the cyst walls (40). It was observed that anti-gGlcT1 morpholino oligonucleotide, which inhibited gGlcT1 activities in trophozoites and cysts, was found to block ESV biogenesis completely (Fig. 2 A). However, ESV synthesis by encysting cells that were transfected with scrambled morpholino oligonucleotide (labeled as control (scrambled) in Fig. 2 A) was not affected at all. To further elucidate the role of gGlcT1 in ESV biogenesis, we overexpressed the *gglct1* gene in trophozoites and subjected them to encystation. Fig. 2 B shows that gGlcT1 overexpression produces aggregated and enlarged ESVs as compared with control cells that were transfected with empty plasmid. This indicates that the modulation of gGlcT1 activity either by knockdown or overexpression affects ESV biosynthesis in *Giardia*. The specificity of anti-cyst antibody used in this study is shown in Fig. 2 C, which reveals that this antibody recognizes cyst wall proteins (CWP1, CWP2, and CWP3) that are synthesized during encystation.

Fig. 3 A demonstrates that gGlcT1 activity in overexpressed trophozoites increased ~3-fold as compared with the activity present in control trophozoites (i.e. specific activities increased from 0.5 pmol/min/mg protein in control to 1.4 pmol/min/mg protein in gGlcT1-overexpressing cells). Knockdown by anti-gGlcT1 morpholino oligonucleotide inhibits gGlcT1 activity in trophozoites by ~50% (from 0.5 to 0.24 pmol/min/mg protein). Furthermore, the rescue experiment (i.e. transfecting gGlcT1-overexpressing trophozoites with anti-gGlcT1 morpholino oligonucleotide) lowered the gGlcT1 activity by ~40%. Fig. 3 B demonstrates that overexpression of gGlcT1 in nonencysting trophozoites causes an increased synthesis of NBD-GlcCer, further suggesting that gGlcT1 activity is directly linked to GlcCer synthesis in *Giardia*.

Next, we asked whether gGlcT1 modulation by overexpression and knockdown also affects the GSL profiles in *Giardia*. For this, lipids were extracted from overexpressed, knockdown, and rescued trophozoites and subjected to ESI-MS/MS analysis as described under “Experimental Procedures.” Using total ion map and precursor ion scans, we identified both neutral and acidic GSLs that were present in control and gGlcT1-modulated trophozoites. Among the neutral lipids, mono-, di-, and tri-hexosylceramides were identified and found to be altered by gGlcT1 modulation. Although gGlcT1 overexpression increased the amount of mono-hexosylceramide by ~28-fold, the effect was somewhat less (~2-fold) on di-hexosylceramides. Interestingly, no effects of gGlcT1 overexpression were observed in the case of tri-hexosylceramides (Fig. 3 C). gGlcT1 knockdown, however, elevated the level of mono-hexosylceramides (~5-fold), and the rescue experiment had no effect, i.e. same as the knockdown (Fig. 3 C). Knockdown also decreased the levels of di- and tri-hexosylceramide slightly, and rescue treatment appeared to reverse these effects (Fig. 3 C). The anal-

FIGURE 2. Knockdown and overexpression of gGlcT1 affects ESV biogenesis. A, Giardia trophozoites were transfected with anti-gGlcT1 morpholino oligonucleotide (50 μM) (23) and subjected to encystation for 24 h before ESVs were analyzed by confocal microscopy. Control trophozoites were transfected with a scrambled morpholino oligonucleotide sequence (50 μM) supplied by the manufacturer. Discrete ESVs are visible in control cells but are not present in anti-gGlcT1 morpholino-transfected (i.e. gGlcT1 knockdown) cells. Arrows show ESVs, and arrowheads indicate nuclei. Bar, 10 μm. B, gGlcT1-overexpressing (+gGlcT1) trophozoites were subjected to encystation for 24 h, and ESVs were analyzed as described above. gGlcT1 overexpression causes an aggregation/enlargement of the ESVs when compared with control cells that were transfected with empty plasmids. Arrows show ESVs, and arrowheads indicate nuclei. Bar, 10 μm. Inset shows the magnified images of ESVs. C, immunoblot analysis reveals that anti-cyst antibody recognizes cyst-specific proteins (i.e. CWP1, CWP2, and CWP3) expressed during encystation. Troph denotes trophozoites; Pre-En indicates pre-encysting trophozoites; 6 h denotes 6-h encysting trophozoites; 12 h denotes 12-h encysting trophozoites; 24 h gGlcT1-knockdown 24-h encysting trophozoites; 48 h indicates 48-h encysting trophozoites.
Giardial GlcT1 Activity and Cyst Viability

Because ESV synthesis is linked to gGlcT1 activity (Fig. 2), and the fact that PPMP blocks encystation (17, 18), we asked if the modulation of gGlcT1 activity (shown in Fig. 3A) also regulates ESV biogenesis. Fig. 4A, panels b and c, demonstrates that although gGlcT1 overexpression induces the synthesis of aggregated and enlarged ESVs, knockdown of gGlcT1 activity by anti-gGlcT1 morpholino oligonucleotide completely blocks ESV synthesis. More interestingly, the rescue experiment reverses the effects of overexpression and knockdown and generates ESVs (Fig. 4A, panel d) that are comparable with control cells (Fig. 4A, panel a). Again, Fig. 4A, panel e, represents ESVs generated by encysting cells that were treated with scrambled morpholino oligonucleotide, which shows the similar pattern of ESVs found in nontransfected control encysting cells shown in Fig. 4A, panel a. Analysis of individual ESVs is shown in Fig. 4B, demonstrating that gGlcT1 overexpression increases the perimeters and areas of ESVs by ~6- and ~10-fold, respectively. The perimeter and area analyses of individual ESVs were carried out with the help of Zeiss Zen 2009 software (Carl Zeiss) as shown in Fig. 4B.

Anti-gGlcT1 Morpholino Oligonucleotide Does Not Interfere with the Growth and Replication of Trophozoites

PPMP is a common inhibitor of GlcT1 enzyme that has been extensively used to evaluate GlcCer functions in various organisms (41). It has been reported that PPMP blocks replication and cytokinesis of giardial trophozoites and is thereby thought to inhibit the encystation and cyst production in culture (18, 26). We have shown earlier that PPMP interferes with the formation of cysts when it is added in the culture medium during encystation (17). Because our current results show that anti-gGlcT1 morpholino oligonucleotide inhibits gGlcT1 activity and ESV biogenesis (Figs. 3 and 4), and because the activity of this enzyme is low in trophozoites and up-regulated in encysting cells, we thought that the effect of PPMP-inhibiting replication and cytokinesis on nonencysting trophozoites might not occur via GlcCer synthesis, as thought previously (18, 26). In fact, there are reports in which PDMP (an analog of PPMP) has been shown to block cell cycle progression in mammalian cells by inhibiting cyclin-dependent kinases, which could be independent of GlcT1 inhibition and ceramide accumulation (42). To address this possibility in Giardia, trophozoites were transfected with morpholino oligonucleotide (anti-gglct1), and the growth was measured as shown in Fig. 5A. Side by side, the growth of the trophozoites in the presence of PPMP (10 μM) was also conducted, and the results showed that although PPMP affects the growth of trophozoites, anti-gGlcT1 morpholino exhibits no effects (Fig. 5A). Fig. 5B, panel b, demonstrates that PPMP, as shown earlier (18, 26), blocks the replication of trophozoites and generates undivided doublet cells. In contrast, anti-gGlcT1 morpholino did not affect the replication of trophozoites (Fig. 5B, panel c). When morpholino-transfected, gGlcT1-knockdown trophozoites were treated with PPMP, the formation of undivided doublets was observed again (Fig. 5B, panel d). The analysis revealed that although ~25% of trophozoites form undivided doublets after PPMP treatment, less than ~5% of doublets was observed in control and anti-gGlcT1 morpholino-treated trophozoites (Fig. 5C). The com-

FIGURE 3. Modulation of gGlcT1 activity by overexpression and knockdown. A, stable Giardia cell lines overexpressing GlcT1 enzyme (designated as +GlcT1) were generated by transfecting trophozoites with pNT5-gctl (tagged with a small peptide, called AU1) plasmid as described under “Experimental Procedures.” Overexpression increased the synthesis of gGlcT1 activity by ~3-fold, which could be reduced by transfecting the overexpressed cells with anti-gGlcT1 morpholino oligonucleotide (i.e. gGlcT1-rescued cells). The enzyme activities in gGlcT1-overexpressing, -knockdown, and -rescued cells were measured and the results are presented in mean values ± S.D. of three separate experiments (**p < 0.01). B, synthesis of NBD-GlcCer by control and gGlcT1 overexpressed (+gGlcT1) trophozoites. Fractions containing NBD-GlcCer were extracted and analyzed as described under “Experimental Procedures,” dried under N2, and re-dissolved in chloroform. 10 μl of sample was spotted in each lane and visualized under a UV lamp. It was noted that gGlcT1-overexpressing cells take up more ceramides than the control cells. Although the experiments were carried out twice separately with different cell preparations, results (TLC) shown here are from a single study. C, ESI-MS/MS analysis of GSLs. GSLs from control (containing empty plasmid) with gGlcT1-knockdown (KD), overexpressed (+GlcT1), and rescued trophozoites were extracted and analyzed as described under “Experimental Procedures.” Mono-hexosylceramide (HexCer), di-hexosylceramide (di-HexCer); tri-hexosylceramide (tri-HexCer); the results (pmol/106 cells) presented here are the mean of fold changes (compared with control trophozoites) ± S.D. of three technical replicates (*p < 0.05; **p < 0.001). D, trophozoites expressing AU1-tagged gGlcT1 were monitored by reacting with anti-AU1 antibody followed by labeling with FITC-conjugated anti-mouse antibody and examination under an immunofluorescence confocal microscope. The figure shows that overexpressed gGlcT1-AU1 (shown as +gGlcT1 in the figure) forms structures that are granular, aggregated, and localized throughout the cytoplasm and, to some extent, in the perinuclear regions. However, the overexpressed gGlcT1 granules are also localized in the ventral groove and lateral shield areas of the trophozoites. White arrowheads indicate DAPI-stained nuclei (control) and long yellow arrowheads denote ventral groove (vg), lateral shield (ls), and plasma membrane (pm) in overexpressed trophozoites. Bar, 10 μm.
FIGURE 4. Modulation of gGlcT1 activity affects ESV biogenesis in Giardia. A, ESV production was monitored in the following: panel a, control; panel b, GlcT1-overexpressing (+gGlcT1); panel c, antisense morpholino oligonucleotide-treated gGlcT1-knockdown cells; panel d, rescued cells, and panel e, cells transfected with scrambled morpholino oligonucleotides. Arrows indicate ESVs, and arrowheads denote nuclei. Bar, 10 μm. B and C, changes of perimeters and areas of ESVs are shown in the bar graphs. The analyses were carried out by measuring the perimeters and the areas of individual ESVs using Zeiss Zen 2009 confocal software. *, p < 0.05; **, p < 0.01.

FIGURE 5. Effects of PPMP and anti-gGlcT1 morpholino on growth and replication of trophozoites. A, assessing the growth of trophozoites. Approximately 2 × 10^4 cells were inoculated into 4-ml tubes containing TYI-S-33 medium, pH 7.1, supplemented with adult bovine serum and bile. Trophozoites were allowed to grow for 35 h (the doubling time of trophozoites is ~7 h) and were counted under a microscope. Each experiment was carried out in triplicate, and the experiment was repeated three times. B, differential interference contrast confocal images show the effects of anti-gGlcT1 morpholino oligonucleotide and PPMP on cell division and cytokinesis. Nuclei were stained with DAPI. Control, panel a; 10 μM PPMP, panel b; knockdown with morpholino, panel c; morpholino + PPMP cells, panel d. C, quantitative assessment (e.g., microscopic counts) of doublet trophozoites that are produced by control, PPMP-treated, anti-gGlcT1 morpholino-induced knockdown trophozoites (knockdown) and knockdown (KD) trophozoites + PPMP. At least 100 singlet, doublet, and triplet (the number of undivided triplet trophozoites were small and therefore are not shown in the figure) trophozoites from 10 different fields were viewed and counted under the Zeiss LSM 700 confocal microscope using Zen 2009 software. The percentage values of doublet cells were calculated based on the total number of singlet, doublet, and triplet cells. *, p < 0.05; **, p < 0.01.
Regulated Expression of gGlcT1 Is Critical for Maintaining Cyst Viability

The production of viable cysts is essential for *Giardia* to establish infection in the small intestine of humans. Earlier reports suggested that cyst morphology is directly correlated with cyst viability, and only viable cysts of *Giardia* produce infection in mice (43, 44). Gillin *et al.* (21) reported that *in vitro*-derived *G. lamblia* cysts are of two kinds, *i.e.* type I and type II. The type I cysts are oval shaped with uniform and refractive cyst walls, being mostly (∼90%) viable. In contrast, type II cysts lack the morphological characteristics of type I, and a majority (∼70%) of them are nonviable. In a heterogeneous cyst population (*i.e.* a population composed of both type I and type II cysts), ∼10–30% of cysts exhibit type I morphology (21, 45). Because we found that the gGlcT1 activity increased during encystation (Fig. 1) and that the modulation of its function (by overexpression and knockdown) regulated ESV biogenesis (Figs. 2 and 4), we asked whether gGlcT1 activity is also important for maintaining the cyst morphology and viability. Therefore, trophozoites from various conditions, *i.e.* control and gGlcT1-overexpressing, gGlcT1 knockdown, and gGlcT1-rescued, were subjected to encystation for 72 h, and the cysts were isolated by centrifugation as described under “Experimental Procedures.” Fig. 6A, panel a, shows control cysts with type I morphology that react with the anti-cyst antibody, which labels the oval-shaped cyst wall. However, this changes significantly in gGlcT1-overexpressing and gGlcT1-knockdown cells (Fig. 6, A, panels b and c). Cysts produced by gGlcT1-overexpressing cells appear to be type II because they are incomplete, clustered, have thin cyst walls, and show minimum reactivity to anti-cyst antibody. Knockdown of gGlcT1 also produces aggregated cryptic cysts; the majority of these cyst-like structures exhibit no reaction to anti-cyst antibody. The rescue treatment, shown in Fig. 6A, panel d, however, produces cysts with well formed cyst walls that react with the cyst antibody. Because the morphology could be an indicative of viability (43, 45), our next goal was to test if the viability of cysts is affected by gGlcT1 overexpression and knockdown. The viable and nonviable cysts were identified by staining with fluorogenic dyes, *i.e.* cell-permeable esterase substrate FDA and the cell impermeant nucleic acid stain PI, which were earlier used by other laboratories to determine the viability of giardial cysts (21, 43, 44). Fig. 6B indicates that although in control samples ∼12% of cysts were viable, only ∼8 and ∼3% of cysts were viable in gGlcT1-overexpressing and gGlcT1-knockdown cells, respectively. Most importantly, the rescue experiment, in which the effect of gGlcT1 was neutralized by the anti-gGlcT1 morpholino analog, recovers the cyst and increases the viability up to ∼10%. This is an important observation and strongly suggests that gGlcT1 activity in *Giardia* not only regulates ESV biogenesis but also maintains the cyst viability. Although it appears that these numbers of viable cysts are somewhat low, our calculation is based on the total number of water-resistant cysts that contain all types of cysts, including type I and type II. Thus, viability estimations, shown here, are within the expected range and in accordance with the report published by Gillin *et al.* (21) and Boucher *et al.* (45).

**gGlcT1 Overexpression Alters Lipid Balance in Trophozoites**

As proposed earlier, *Giardia* trophozoites have limited abilities to synthesize membrane lipids, cholesterol, and fatty acids *de novo* and thus depend on supplies from the small intestines, where the trophozoites colonize (46, 47). Studies suggest that most of the lipids and fatty acids are taken up by this parasite from outside sources and are utilized for energy metabolism and biogenesis of organelles and vesicles (48–50). Because gGlcT1 overexpression induces the synthesis of enlarged vesicles and produces cysts with reduced viability (Figs. 2, 4, and 6), we investigated whether gGlcT1 expression is associated with increased lipid uptake by *Giardia* trophozoites. We asked whether gGlcT1 expression is linked to an increased influx of membrane lipids and whether the intracellular levels of chole-
terol and fatty acid are altered by gGlcT1, which in turn would affect the membrane fluidity and lipid uptake.

**gGlcT1 Overexpression Affects the Internalization and Intracellular Targeting of Fluorescent Lipids**—Using fluorescently labeled (Bodipy or NBD) lipid and fatty acid analogs, we have shown earlier that trophozoites have the machinery to recruit lipids from their environment and target them into specific cellular locations, including the plasma membrane and endomembranes, as well as the cytoplasm (28). For example, ceramide and PG were found to be localized at ER/perinuclear membranes, and PC was incorporated into plasma and flagellar membranes of trophozoites. We found exogenous SM were targeted into nuclear and plasma membranes and PE in the inner layer of the plasma membrane (51, 52). Furthermore, it was shown by us earlier that ceramide is taken up by *Giardia* via a clathrin-dependent pathway and is likely to be regulated by giardial serine palmitoyltransferase, a rate-determining enzyme of SL biosynthesis (17, 49). The internalization of various fluorescent lipid probes, including Bodipy-ceramide, NBD-SM, Bodipy-PG, Bodipy-PC, NBD-PE, and FAST Dil (a common lipid-labeling dye), was studied in control and gGlcT1-overexpressing trophozoites. The results demonstrated that gGlcT1 overexpression increased the uptake and labeling intensities of ceramide, SM, PG, PE, and FAST Dil (Fig. 7A). Interestingly, PE, which is located in the inner plasma membranes, migrates inside the cells and is concentrated in the ER/perinuclear membranes (Fig. 7, A, panels i and j), whereas PC, another major lipid of the plasma membrane, is not affected by gGlcT1 (Fig. 7A, panels e and f). The labeling by FAST Dil (which labels the lipids in plasma and intracellular membranes) is also increased in gGlcT1-overexpressing cells (Fig. 7A, panels k and l). These results suggest that gGlcT1 activity is responsible for the influx of majority of cellular lipids in *Giardia*.

**gGlcT1 Overexpression Elevates the Intracellular Levels of Cholesterol and Fatty Acid in Trophozoites**—Like other lipid molecules, *Giardia* also has a limited ability to synthesize cholesterol and fatty acids *de novo*, which are obtained from the growth medium. Interestingly, both cholesterol and fatty acids have been shown to be involved in regulating encystation and cyst formation by this waterborne pathogen (48, 53, 54). As gGlcT1 overexpression increased the internalization of fluorescent lipid probes from the medium (Fig. 7), we thought it would be important to examine whether gGlcT1 expression also changes the cellular fatty acid and cholesterol levels. Cholesterol and free fatty acids in control and gGlcT1-overexpressing cells were analyzed by GC-MS. The sterol analysis showed that cholesterol is the major sterol in both control and gGlcT1-overexpressing cells (Fig. 8A), and no other sterols, including cholesteryl esters or ergosterol, were detected (55). It is interesting that gGlcT1 overexpression increased the intracellular level of cholesterol by ~2-fold. Fatty acid analysis (Fig. 8B) revealed that palmitic acid, stearic acid, oleic acid, and linoleic acid were the major fatty acids present in *Giardia* trophozoites and that gGlcT1 overexpression increased the level of these fatty acids by ~20, ~22, ~25, and ~38%, respectively. Thus, as with other lipids as shown in Fig. 7, gGlcT1 expression also increased the uptake of cholesterol and fatty acids by *Giardia* (Fig. 8, A and B).

Because the changing of membrane fluidity may increase lipid uptake by trophozoites, we investigated the possibility that the membrane fluidity could be modified due to the up-regulation of cholesterol and fatty acids by GlcT1, allowing the cells to internalize excess fluorescent lipids as shown in Fig. 7. The membrane fluidity was measured using a fluorescent lipid probe, pyrene decanoic acid, as detailed under “Experimental Procedures.” This pyrene probe usually forms excimer by interacting with membrane components that cause a shift of emission spectrum to a longer wavelength. Therefore, the ratio of monomer to excimer shift could be utilized to measure the change of membrane fluidity (described by the manufacturer, Marker Gene Technologies). Fig. 8C shows that gGlcT1 overexpression did not produce pyrene excimers from monomers. However, the trophozoites treated at 4°C (cold-shock) showed significant changes from monomer to excimer of the pyrene probe (Fig. 8D). This suggests that the fluidity of trophozoite plasma membranes is not affected by gGlcT1 overexpression, although it increased the uptake of lipid, cholesterol, and fatty acids. We speculate that the increased lipid uptake by gGlcT1-
overexpressed trophozoites occurs via receptor- or raft-mediated lipid endocytosis (48, 49) rather than to the change of membrane fluidity.

**DISCUSSION**

In this study, we show that gGlcT1 activity is important in the production of viable cysts by *Giardia* and that it acts by regulating ESV biogenesis and controlling the import of lipids and fatty acids from the environment.

Several strategies were followed to modulate gGlcT1 activity in *Giardia*. First, gGlcT1-knockdown trophozoites were generated (using anti-*gglct1* morpholino oligonucleotide), which reduced the activity of gGlcT1 in both cysts and trophozoites (Figs. 1B and 3A). Second, the *gglct1* gene was overexpressed in trophozoites, and this caused an elevation of enzyme activity by ~3-fold (Fig. 3A). Third, the increased gGlcT1 activity in overexpressed cells was rescued by knocking down the gene using morpholino oligonucleotide, which reduced the excess activity caused by the overexpression of the *gglct1* gene (Fig. 3A). We found that the modulation of gGlcT1 activity directly correlated with ESV biogenesis (Fig. 4). When the excess gGlcT1 activity in gGlcT1-overexpressing cells was normalized by introducing anti-*gglct1* morpholino oligonucleotide, ESV synthesis was restored and appeared to be normal as judged by confocal microscopy as well as by analyzing the perimeter and area of individual ESVs (Fig. 4). These results suggest that gGlcT1 directly influences the secretion and maturation of ESVs, which are critical for cyst production/viability. This postulation can be further supported by our cyst experiment, in which the rescue treatment generated classical oval-shaped cysts with thick cyst walls that are similar in morphology and viability to control cysts (Fig. 6).

To identify the possible mechanism how gGlcT1 regulates ESV biogenesis and cyst production, we investigated the overall lipid balance in gGlcT1-overexpressing trophozoites. Our results showed that the elevated gGlcT1 activity caused an increased uptake of mono-hexosylceramides, fluorescent lipids, cholesterol, and fatty acids (Figs. 3C, 7, and 8). The rising levels of mono-hexosylceramide in gGlcT1-overexpressed cells can also be caused by the *de novo* synthesis of these molecules (catalyzed by an active gGlcT1 enzyme) in addition to the increased uptake from the growth medium (Fig. 3C). Therefore, it is likely that *Giardia* uses its gGlcT1 enzyme to maintain an overall lipid balance by importing a majority of lipids from out-
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side and synthesizing a selective few (50–54). We propose that the controlled expression of gGlcT1 is critical for lipid internalization in trophozoites because overexpression or knocking down of this enzyme affects ESV biogenesis and cyst viability. The observation that gGlcT1 regulates the overall lipid balance/homeostasis in Giardia was further supported by the report that GlcT1 overexpression influences several body fat storage genes in Drosophila (56) and also that the interactions between SL and other branches of the lipid metabolic pathways are essential for maintaining lipid homeostasis in mammalian cells (57, 58).

As far as the effect of PPMP on Giardia trophozoites is concerned, it has been reported (18, 26) that this inhibitor interferes with the replication and cytokinesis of trophozoites and thereby blocks encystation. This action of PPMP has been attributed to GlcT1 inhibition and intracellular ceramide accumulation (26). In contrast, we observed that anti-gglct1 morpholino oligonucleotide treatment did not reduce the growth and replication of Giardia trophozoites (Fig. 5), but it did inhibit ESV biogenesis and cyst production when subjected to encystation. Although we did not measure the intracellular concentration of ceramide after PPMP treatment, there is evidence that another GlcT1 inhibitor, PDMP, can influence the cell cycle regulation by inhibiting cyclin-dependent kinases and not exclusively by inhibiting GlcCer synthesis. Detailed analysis revealed that PDMP treatment caused a reversible decrease in the activity of cyclin-dependent kinases (i.e. cdk2 and p34cdc2), which led to cell cycle arrest (42, 59, 60). Thus, it is possible that PPMP-induced blocking of trophozoite replication and cytokinesis could be an off-target effect that is caused by the inhibition of giardial cell cycle proteins and kinases (61) rather than the inhibition of GlcT1 and accumulation of ceramide. However, more in-depth experiments should be undertaken to resolve this issue.

Although in our study it is not clear why cyst stage gGlcT1 is not inhibited by PPMP (Fig. 1), Stefanić et al. (26) reported that Giardia synthesizes di- and tri-hexosylceramides in encysting cells and cysts. Hillig et al. (62) also reported that GCS in plant cells shows broader substrate affinities and functions as a sterol glucoside synthase. Interestingly, gGlcT1 and plant GCS are remarkably similar as far as the amino acid identity and sequence motifs are concerned (26), and therefore, like plant GCS, gGlcT1 may exhibit broader substrate preferences and catalyze the synthesis of di- and tri-hexosylceramides as shown in Fig. 3C. We observed that knockdown of gGlcT1 activity by anti-gGlcT1 phosphorolo also elevated the levels of mono-hexosylceramides over control trophozoites, which could probably be due to membrane damage caused by the electroporation that allows cells to uptake excess mono-hexosylceramides from the medium (Fig. 3C). This conclusion is based on our observation that the mono-hexosylceramide level also increases in trophozoites that are transfected with scrambled morpholino oligonucleotides (data not shown).

Earlier studies identified and characterized an enzyme, i.e. cyst wall synthase (N-acetylgalactosaminyltransferase), which is involved in the formation of insoluble N-acetyl-galactosamine homopolymer, a major component of the giardial cyst wall (63). It is likely that cyst gGlcT1 interacts with cyst wall synthase and facilitates the process of cyst production. However, detailed experiments are necessary to characterize the GlcT1 activity in cysts and the classes of complex glycosphingolipids that are formed during encystation. Moreover, there are instances where GSLs have been shown to be involved in maintaining the growth and morphology of mammalian cells. For example, GSL-deficient B16 melanoma cells exhibit altered morphology and slower growth rates (64). GlcCer synthesis is also necessary for preserving the virulence of infective fungal cells (65, 66). Thus, based on these observations, we propose that gGlcT1, like fungal cells, is an important pathogenic determinant of this parasite and should be targeted for developing new drugs to control giardiasis. The result that gGlcT1 is critical for maintaining cyst viability shown in this study should open a new possibility for investigation of whether gGlcT1 knockdown cysts with reduced viability can also be utilized as a live vaccine candidate to control giardiasis.

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