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Glucosylceramide transferase in *Giardia* preferentially catalyzes the synthesis of galactosylceramide during encystation

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Abstract

The stage differentiation from trophozoite to cyst (i.e., encystation) is an essential step for *Giardia* to survive outside its human host and spread the infection via the fecal-oral route. We have previously shown that *Giardia* expresses glucosylceramide transferase 1 (GlcT1) enzyme, the activity of which is elevated during encystation. We have also reported that blocking the activity of gGlcT1 interferes with the biogenesis of encystation-specific vesicles (ESVs) and cyst viability in *Giardia*. To further understand the role of this enzyme and how it regulates encystation, we overexpressed, knocked down, and rescued the giardial GlcT1 (gGlcT1) gene and measured its enzymatic activity in live parasites as well as in isolated membrane fractions using NBD-ceramide and UDP-glucose or UDP-galactose. We observed that gGlcT1 is able to catalyze the synthesis of both glucosylceramide (GlcCer) and galactosylceramide (GalCer), however the synthesis of GalCer is 2–3 fold higher than of GlcCer. Although both activities follow Michaelis–Menten kinetics, the bindings of UDP-glucose and UDP-galactose with the enzyme appear to be non-competitive and independent of each other. The modulation of gGlcT1 synthesis concomitantly influenced the expression cyst-wall protein (CWP) and overall encystation. We propose that gGlcT1 is a unique enzyme and that *Giardia* uses this enzyme to synthesize both GlcCer and GalCer to facilitate the process of encystation/cyst production.

Graphical abstract

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Keywords

Giardia lamblia; Encystation; Sphingolipid; Glucosylceramide Transferase; Glucosylceramide; Galactosylceramide; Anti-sense morpholino oligonucleotide

1. INTRODUCTION

Giardia lamblia is an enteric parasite and a major cause of waterborne diarrhea, worldwide [1]. Spread via the fecal-oral infective route, *Giardia* has been implicated in symptoms such as malabsorption, malnutrition, fatigue [2], and in severe cases the parasite may cause stunted growth in children [3]. *Giardia* exists in two morphologic forms: active trophozoites that colonize in the upper part of the small intestine (the duodenum and jejunum areas) and the infective cyst, which is defecated and infects a new host. The trophozoites undergo stage differentiation to cyst (called "encystation") in the lower small intestine where they begin to construct cyst walls that are composed of proteins, glycoproteins, and polysaccharides [4-6]. During cyst formation, unique encystation specific vesicles (ESVs, average perimeter, 1–2 μ m) are synthesized to transport cyst-wall proteins (CWP1, CWP2, CWP3) from the endoplasmic reticulum (ER) to the plasma membrane of trophozoites and lay down the cyst wall [7]. Without the formation of a rigid cyst wall, *Giardia* will not survive in the environment and transmit infection from one host to another.

[In recent years, sphingolipids (SLs) have been shown to play an important role during the growth and encystation of *Giardia* [8]. Only five SL genes are present in this parasite (www.GiardiaDB.org) including two subunit genes of serine palmitoyltransferases (*gspt-1* and *gspt-2*), glucosylceramide transferase (*gglct1*), and two acid sphingomyelinase genes (*gsmase 3b* and *gsmase B*). This suggests that *Giardia* has a limited ability to synthesize sphingolipids de novo. Although major SLs such as sphingosine, ceramide, glycosphingolipids, and sphingomyelin are present in *Giardia*, they are mostly acquired from the medium. However, *Giardia* has the ability to synthesize monohexosyl- and dihexosylceramide during encystation]. We have reported earlier that giardial glucosylceramide transferase-1 (gGlcT1; also called GlcCer synthase or GCS) is upregulated during encystation [9]. GlcT1 catalyzes the synthesis of GlcCer by transferring glucose from UDP-glucose to a ceramide molecule. Interestingly, ceramide is not synthesized by *Giardia* de novo and proposed to be acquired from the growth medium [10-12]. During encystation,

the ceramide changes its position and localizes in parallel perinuclear cisternae that appear to be Golgi-like structures (or ESVs) [13, 14]. This suggests the possibility that *Giardia* uses exogenous ceramide for signaling purposes and as substrate for the gGlcT1 enzyme to produce GlcCer [9, 15]].

Here we show for the first time that gGlcT1 has a dual substrate enzyme and is able to utilize both UDP-glucose (UDP-Glc) and UDP-galactose (UDP-Gal). However, UDP-Gal is preferred over UDP-Glc. Furthermore, we demonstrate that the modulation of gGlcT1 activity influences the expression of cyst-wall protein (CWP) and overall encystation process.

2. MATERIALS AND METHODS

2.1. Materials

The majority of chemicals and supplies were purchased from Sigma-Aldrich (St. Louis, MO). N-[7-(4nitrobenzo-2-oxa-1, 3-diazole)] conjugated ceramide (NBD-ceramide), Prolong^R Gold antifade reagent and customized antibody (polyclonal) against giardial glucosylceramide transferase (anti-gGlcT1) was obtained from Thermo Fisher Scientific, Inc. (Waltham, MA). Cyst antibody (monoclonal) was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX). AU1 sepharose beads were obtained from Covance, Inc. (Princeton, New Jersey). TLC plates were purchased from EMD Millipore (Billerica, MA). Fluorescein labeled Wheat Germ Agglutinin (WGA) was obtained from Vector Labs (Burlingame, CA).

2.2. Parasite, overexpression, knockdown, and rescue

G. lamblia trophozoites (strain WB, ATCC No. 30957) were cultured and encysted as previously described [16, 17]. gGlcT1 overexpression with AU1 (a six-amino acid containing peptide i.e., DTYRYI) epitope in *G. lamblia* and the knockdown of the *gglt1* gene (encoding gGlcT1 enzyme) was carried out with the help of anti-*gglt1*-morpholino oligonucleotide (5'-CTAAGGAGAGAGTCAACCCGTCCAT-3') as we have previously described [15]. For rescue, gGlcT1 [overexpressing] trophozoites were transfected with anti-*gglt1*-morpholino oligonucleotide, recovered for 20 h [15] and then subjected to encystation and measuring enzyme activities as described below.

2.3. NBD-ceramide metabolism

Glycosylation of NBD-ceramide by *Giardia* was performed following a previous reported method [15, 18] with slight modifications. Non-encysting and encysting trophozoites were harvested and 1×10^7 cells were transferred to a 1.5-mL microcentrifuge tube and starved in 1.5-mL DMEM (Dulbecco's Modified Eagle Medium-without serum supplementation) for 1 h at 37 °C. Following starvation, NBD-ceramide (15 µM) was added directly to the suspension and cells were allowed to uptake and metabolize the NBD-ceramide for 3 h at 37 °C in the dark. Cells were collected by centrifugation at 1500g for 15 min and glycosphingolipids (GSLs) were extracted as described elsewhere [15]. Cells were resuspended in 1.3 mL of water, 1.3 mL of chloroform, and 1.3 mL of acetic acid/methanol (1:50 v/v), vortexed for 1 min and centrifuge at 3000g for 10 min. The organic phase was

collected and transferred to a glass vial. The extraction was repeated one more time and the organic phases were pooled together and dried under N₂ conditions. Dry samples were resuspended in 80 μ L chloroform/methanol (1:1 v/v) and stored at -20°C .

2.4. Membrane preparation and enzymatic activities

G. lamblia were cultured in a 75-cm² flask, harvested using a cell scraper, and washed with PBS. Cells [$\sim 1 \times 10^8$] were subjected to hypotonic shock with 50 μ L of hypotonic buffer (5 mM HEPES-pH7.4, 0.5 mM EDTA), for 30 min at 4°C . Cells were then re-suspended in 300 μ L of lysis buffer (50 mM HEPES, pH7.4, 250 mM sucrose, 25 mM KCl and 5 mM MnCl₂, 10 μ M E64, and a protease inhibitor cocktail) and sonicated in a cold waterbath sonicator for 10 min, passed through a 27-G X 1 $\frac{1}{4}$ needle 20 times; these steps were repeated one more time. The homogenate was centrifuged for 5 min at 500g, and the supernatant (S₅₀₀) was set aside. The pellet (P₅₀₀) was resuspended in 300 μ L of lysis buffer, sonicated, passed through a syringe, and centrifuged for 5 min at 500g. The first and second supernatants (S₅₀₀) were combined and centrifuged for 30 min at 15 000g. The pellet was resuspended in 100 μ L lysis buffer and used as an enzyme source. All procedures were carried out at 4°C . gGlcT1 activity was assayed by measuring the transfer of sugar moieties from UDP-Glc or UDP-Gal to NBD-ceramide and monitoring the formation of NBD-GlcCer and NBD-GalCer [19] with slight modifications. Standard reaction mixture contained 50 mM HEPES-pH 7.4, 250 mM sucrose, 25 mM KCl, 5 mM MnCl₂, 1 mM UDP-Glc, and the enzyme source (5 μ g protein/assay mixture). The reaction was initiated by the addition of 5 μ M NBD-ceramide in a total volume of 50 μ L reaction mixture and incubated while shaking for 60 min at 37°C . To examine whether gGlcT1 enzyme also catalyzes the formation of galactosylceramide, UDP-Gal (0.5 mM) was used instead of UDP-Glc as a donor and the reaction was carried out for 10 min at 37°C . [We also used GDP-mannose (0.5-1.0 mM) under the same condition to test if gGlcT1 can synthesize mannosylceramide (Man-Cer)]. Reactions were stopped by placing the tubes on a -80°C cool rack. The initial velocities of GlcT1 activity were performed in the buffer describe at various concentrations of the UDP-Glc (0.5 μ M-2.0 mM) or UDP-Gal (0.5 μ M-0.5 mM UDP-Gal).

2.5. Immunoprecipitation using AU1 antibody

G. lamblia were cultured in a 75-cm² flask, encystation was induced for 20 h, and cells [$\sim 10^8$] were harvested using a cell scraper and washed with PBS. Cells were resuspended in 500 μ L of PBS containing protease inhibitors and the suspension was sonicated using a 1/8" microtip with 5 short bursts of 20 sec and 30 sec of cooling during intervals. The lysate was cleared at 3000g for 2 min and 200 μ L of AU-sepharose beads were added to the lysed and incubated overnight at 4°C . Immunoprecipitates were washed four times with 500 μ L PBS and the last wash was performed with the enzymatic reaction buffer (see above). Immunoprecipitates were resuspended in 200 μ L of enzymatic reaction buffer and aliquots of 40 μ L were used to test the GlcT1 enzymatic activity.

2.6. Glycosphingolipid separation by thin-layer chromatography

Samples from enzymatic activity (10 μ L) without lipid extraction or extracted glycosphingolipids (20 μ L) were spotted directly to silica gel 60 TLC plates (Merck), and

resolved using 2-propanol/15 M ammonium hydroxide/ethyl acetate/water (75:5:5:25, v/v/v/v) as a mobile phase [20]. Fluorescent chromatograms were captured by using Gel Doc™ XR+ System (BIORAD) and fluorescent pixels were quantified using ImageJ software (NIH). To construct the standard curve, NBD-GlcCer and NBD-GalCer were spotted onto the TLC plate and resolved together with the samples. The amount of NBD-GlcCer and NBD-GalCer was quantified with their respective standard curves.

2.7. SDS-PAGE and immunoblotting

Protein samples (10 µg) were separated by 12% SDS-PAGE and transferred to a PVDF membrane by a CAPS transfer buffer (10 mM CAPS, pH 11 and 10% methanol) [21]. Non-specific antibody binding sites were blocked in TBS-T (25 mM Tris-pH 7.5, 500 mM NaCl, 0.1% Tween 20) containing 1% BSA. For the detection of gGlcT1, a custom rabbit polyclonal antibody (dilution 1:500) was used. A mouse monoclonal anti-cyst antibody (dilution 1:3000) detected giardial cyst wall protein-1 (gCWP-1). Giardial protein-disulfide isomerase-2 (gPDI-2) rabbit polyclonal antibody (a gift from Dr. Frances Gillin, UC-San Diego) (dilution 1:350000) was used as a loading marker. The appropriate HRP-labeled secondary antibodies (dilution 1:40000) detected bound antibodies and ECL solution (100 mM Tris-pH 8.8, 2.5 mM luminol, 0.4 mM 4IPBA, 2.6 mM H₂O₂ [22] was used for the detection of protein bands.

2.8. Confocal microscopy

Trophozoites and encysting cells were harvested and fixed with 4% paraformaldehyde for 10 min. After washing with PBS, the cells were permeabilized with 0.1% Triton X-100, washed with PBS, and blocked with 5% NGS for 20 min at room temperature. The 5% NGS was replaced with cyst antibody (monoclonal, 1:100) and 5 µg/mL of Wheat Germ Agglutinin (WGA) in 1% NGS and kept at 4°C overnight. The cells were washed with PBS, incubated with secondary antibody (1:500, Alexa Fluor 594) for 1 h, and subsequently mounted with Prolong^R Gold antifade reagent. Samples were analyzed in a confocal microscope (Carl Zeiss Laser Scanning System LSM 700).

[2.9. Bioinformatic analysis

The hidden Markov model (HMM) was used to identify potential galactosylceramide (EC: 2.4.1.45) and glucosylceramide transferases (EC: 2.4.1.80) sequences. An initial alignment was performed using amino acid sequences from the following KEGG entries—i.e., K04628 and K00720 using MUSCLE aligner [23]. A profile HMM was constructed for each enzyme and searched against the *Giardia* proteins using the software package HMMER 3.0 [24]. Candidate homologs (e-value <0.05) were aligned to known enzymes in order to verify conserved domains and sequence similarity].

2.10. Statistical analysis

Statistical analyses were performed using Sigmaplot 12.5 (Systat Software). Data sets were processed using ANOVA with Bonferroni corrections. More details are shown on Fig. legends.

3. RESULTS

3.1. Glycosylation of NBD-ceramide by live *Giardia*

It has been previously reported that gGlcT1 enzyme activity is induced during encystation and thought to be linked to ESV biogenesis and cyst production by *Giardia* [15]. To better understand the types and classes of hexosylceramides that are produced by gGlcT1, trophozoites and encysting cells were allowed to uptake a fluorescent analogue NBD-ceramide. After labeling with NBD-ceramide, cells were collected, washed, and lipids were extracted followed by a separation on TLC plates. Fig. 1A compares the glycosylation profile of NBD-ceramide in non-encysting and encysting *Giardia*. By comparing their migrations, the products were identified with standard NBD-ceramide, NBD-GlcCer, NBD-GalCer and NBD-LacCer. Results show that while no glycosylation of NBD-ceramide was noted in non-encysting trophozoites, the synthesis of NBD-GlcCer and NBD-GalCer was observed in encysting (24 hours post-induction of encystation or 24 h.p.i.e) cells and gGlcT1 overexpression and knockdown (using anti-*gglt1* morpholino oligonucleotide) modulated the syntheses of both NBD-GlcCer and NBD-GalCer. Fig. 1B shows the quantitative assessment of each glycolipid produced under various conditions previously described. Results indicate that NBD-GalCer and NBD-GlcCer synthesis in encysting cells are comparable (6.1 vs. 4.5 pmol/h $\times 10^7$ cells). Knockdown, on the other hand, reduces the expression of both glycolipids, NBD-GalCer and NBD-GlcCer (4.7 vs 1.4 pmol/h $\times 10^7$ cells). The synthesis of these two glycolipids increases in gGlcT1-overexpressed cells (encysting) and the level of NBD-GalCer are ~3-fold higher than NBD-GlcCer (29.5 vs. 10.5 pmol/h $\times 10^7$ cells). These studies imply that the enzyme gGlcT1 in encysting *Giardia* has the ability to glycosylate NBD-ceramide to NBD-GlcCer and NBD-GalCer, and the production of NBD-GalCer is higher than NBD-GlcCer.

3.2. Glycosylation of NBD-ceramide by isolated membranes

Since the overexpression and knockdown of the *gglt1* gene modulates ceramide glycosylation in live cells (Fig. 1), we hypothesized that the same enzyme (i.e., gGlcT1) can use both UDP-Glc and UDP-Gal as substrates. To test our hypothesis and to better characterize gGlcT1, we measured activities in whole cell extract (S_{500}) and crude membrane fractions (P_{15000}). Fig. 2A shows a representative chromatogram of NBD-GlcCer and NBD-GalCer catalyzed by gGlcT1 from whole cell extract (S_{500}), crude membrane fraction (P_{15000}) and soluble fraction (S_{15000}). The amount of NBD-ceramide used in the current study was determined from a separate experiment of fixed 2-mM UDP-Glc and varying concentration of NBD-ceramide (0.5 to 7.5 μ M, limited substrate) vs. time (0 to 120 min, data not shown). gGlcT1 activities were measured by independent reactions—i.e., UDP-Glc (1 mM) or UDP-Gal (0.5 mM) and NBD-ceramide (5 μ M) as detailed in the method section—and the formation of hexosylceramides was evaluated by TLCs. Results demonstrate that the formation of NBD-GlcCer or NBD-GalCer depend on the presence of respective substrates (UDP-Glc or UDP-Gal) in the assay mixture. [However, when GDP-mannose was used as a mannosyl donor, no NBD-ManCer formation was observed (not shown)]. Interestingly, a small amount of NBD-LacCer was synthesized by S_{15000} supernatant fraction. Fig. 2B demonstrates that gGlcT1 activity and the synthesis of NBD-GlcCer and NBD-GalCer are modulated by overexpression, knockdown, and rescue of the

gglct1 gene in encysting *Giardia*. For example, while *gglct1* overexpression stimulates GlcT1 activities with both substrates in encysting (24 h.p.i.e) parasite (0.279 vs, 0.761 nmol/min.mg. protein for NBD-GlcCer; 1.292 vs. 1.668 pmol/min/mg. protein for NBD-GalCer), knockdown of the *gglct1* gene reduces these activities significantly when compared with control (non-transfected) encysting cells. Rescuing the effect of gGlcT1 by transfecting gGlcT1-overexpressed cells with anti-*gglct1* morpholino oligonucleotide decreased the activity in gGlcT1-overexpressed (+gGlcT1) cells (~23% for NBD-GlcCer and ~15% for NBD-GalCer. Compared with NBD-GlcCer, NBD-GalCer production is consistently higher in control (~5 fold) and +gGlcT1 (~2 fold) encysting cells. To confirm that both NBD-GlcCer and NBD-GalCer products were catalyzed by gGlcT1 enzyme and increased in +gGlcT1 cells, gGlcT1 was immunoprecipitated from +gGlcT1 encysting cells using AU1 antibody bound to sepharose as described in the method section followed by measuring the enzyme activity as shown in Fig. 2C. Results indicate that NBD-GlcCer is produced when UDP-Glc is used. Likewise, NBD-GalCer is the major product when the substrate is UDP-Gal. The gGlcT1 activity (pmol/min) from IP samples is shown Fig. 2D. These studies support our hypothesis that *gglct1* overexpression and knockdown influences the expression of gGlcT1 in encysting cells and catalyzes the formation of NBD-GlcCer and NBD-GalCer, respectively.

3.3. The kinetic analysis and substrate competition

GlcT1 is an important enzyme of the sphingolipid (SL) metabolic pathway. While mammalian GlcT1 is localized in the Golgi complex, in *Giardia* it is located in the ER [25, 26]. Our studies indicate that gGlcT1 is unique because it utilizes both UDP-Glc and UDP-Gal to synthesize respective hexosylceramides (Figs. 1 and 2). To further characterize the differences and similarities between NBD-GlcCer and NBD-GalCer transferase activities, a kinetic analysis of crude membrane fraction (15000×g pellet) was performed and their substrate affinities were compared. Kinetic profiles of gGlcT1 were determined from the Michaelis–Menten plots (Fig. 3A) and the apparent K_m for both UDP-Glc and UDP-Gal were similar (7.1 and 9.2 μM , respectively) but the apparent V_{max} values were different (0.4 and 1.6 nmol/min/mg protein, respectively). Results demonstrate that gGlcT1 has similar affinities for UDP-Glc and UDP-Gal but the rate of transfer of sugar moiety to NBD-ceramide is different. Because the competition among substrates for a specific enzyme or the competition among enzymes for a particular substrate regulates many enzymatic reactions [27]—and since gGlcT1 can utilize both UDP-glucose and UDP-galactose, we wondered if these two substrates compete for the same catalytic pocket of gGlcT1. To test this, we measured gGlcT1 activity with both substrates at the same time. While UDP-Glc concentration was kept fixed at 1 mM, the amount of UDP-Gal varied from 0.05 to 0.5 mM. It was observed that at a higher concentration (0.5 mM), UDP-Gal caused a slight inhibition of NBD-GlcCer synthesis (~20%) but the production of NBD-GalCer increased steadily and reached a maximum (~0.29 nmol/min/mg. protein, ~2 fold higher than UDP-glucose utilization) (Fig. 3B). Likewise, when the activity of gGlcT1 for varied amounts of UDP-Glc (0.05 to 1 mM) was determined in the presence of fixed 0.05 mM UDP-Gal, no inhibition of NBD-GlcCer synthesis was noted and the velocity of reaction reached the plateau at 0.1 mM (Fig. 3C). However, in this condition, gradual increases of UDP-Glc result in a decrease of NBD-GalCer synthesis (90%) due to saturation of the system by UDP-Glc. Taken together,

the enzymatic activity, the enzymatic kinetic, and the dual substrate assays show that UDP-Gal is more preferable to gGlcT1 than UDP-Glc (Figs. 1–3). We speculate that UDP-Glc and UDP-Gal may have separate catalytic sites and their utilization by gGlcT1 is independent. It is also likely that the differential preference for UDP-sugar substrates of gGlcT1 could be due to its localization in different membrane compartments (i.e. ER and plasma membranes) [27, 28].

[3.5. gGlcT1 contains conserved domains of ceramide glucosyltransferase as well as a small domain of ceramide galactosyltransferase]

A regular BLAST database search indicates that gGlcT1 shares maximum homology with ceramide glucosyltransferase (CGlcT) from *Gossypium arborium* (tree cotton, 26.8%) [28, 29] and *Arabidopsis thaliana* (27.8%) [30]. Amino acid sequences of the catalytic motifs of eukaryotic CGlcT such as D1, D2, D3, and (Q/R) *XXRW* are present in gGlcT1 [25, 26]. Interestingly, our results demonstrate that gGlcT1 also contains ceramide galactosyltransferase (CGalT) activity (Figs. 1–3). This finding encouraged us to refine our search tool. We used profile-hidden Markov models of CGlcT (EC 2.4.1.80) and CGalT to search against the *G. lamblia* (strain ATCC 50803/WB clone C6), which identified candidate proteins such as GL50803_11642 (E-val 1.3×10^{-10}), CGlcT, and a low-scoring match with CGalT with a hypothetical protein (GL50803_94224; E-val 0.0024). The sequence analysis of CGlcT reveals the presence of two conserved bipartite NRD2L and NRD2S domains (signature domains for CGlcT [31]) in the N-terminal site of gGlcT1 (Fig. 4). The multiple sequence alignments of the hypothetical protein GL50803_94225 identifies conserved residues (aa 242–267, Fig. 4) of known CGalT [31], which could be responsible for the potential catalytic functions of this enzyme as CGlcT and CGalT. Other features of gGlcT1 (Fig. 4) include a predicted transmembrane domain near the C-terminal site, including a canonical ER retrieval signal [32].]

3.4. Modulations of gGlcT1 activity influence the cyst-wall protein (CWP) synthesis and ESV biogenesis

The process of encystation begins with the biogenesis of ESVs that traffic CWPs to the plasma membranes of trophozoites [33]. During encystation, the dramatic changes that occur in many genes and proteins enable *Giardia* to form hardy water-resistant cysts that can survive outside the human body for months and even years [34, 35]. It has been previously reported that small molecule inhibitors like D-*threo*-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP) and D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP)—both of which are known to inhibit the GlcT1 enzyme—altered the expressions of CWP genes [25, 26]. This indicates that gGlcT1 activity and the syntheses of CWPs are interlinked. Furthermore, Mendez et al. [15] demonstrated that the overexpression and knockdown of gGlcT1 interferes with ESV biogenesis and cyst production. In the current study, we assessed if the gGlcT1 function and CWP expressions are directly related.

Whole cell extract was prepared from encysting and non-encysting cells and analyzed by Western blotting. gGlcT1, CWP1 and gPDI2 were detected using anti-gGlcT1, anti-CWP1 and anti-PDI2 antibodies, respectively, and Fig. 5A is a representative Western blot. While the expressions of both the gGlcT1 and CWP1 proteins are upregulated during encystation

and modulated by overexpression, knockdown, and rescue of the *gglt1* gene, gPDI2 protein level was not affected and was used as loading control. Fig. 5B is a representation of gGlcT1 and CWP1 expression ratios (relative to PDI2) during encystation. Control cells show a remarkable induction of gGlcT1 and CWP proteins during encystation. Likewise, +gGlcT1 cells show an increased expression of gGlcT1 (~3-fold) and CWP1 (~1.8-fold) compared with control encysting cells. Although knockdown of gGlcT1 activity lowers the expression of both gGlcT1 and CWP1 (~80% decrease), rescuing the gGlcT1-overexpressed cells with anti-*gglt1* morpholino oligonucleotide that normalizes the expression of gGlcT1 protein by ~50%. However, it should be noted that the effect of knockdown on the expression of gGlcT1 protein (Fig. 5A, B) is more robust than its (i.e. gGlcT1) enzyme activity (Fig. 1 and Fig. 2). This could be due to the fact that these experiments are different, and therefore the level of changes that were observed by knockdown may not be equal in all three experimental conditions. Furthermore, in the current study, cell lysate and crude membrane fractions (Fig. 2) were employed, which contained other proteins in addition to GlcT1 and that may have reduced the knockdown effects on respective enzymes (i.e. CGlcT and CGalT).

Confocal images of *Giardia* cells show that encystation stimuli induce the biogenesis of ESVs (Fig. 5C). Non-encysting trophozoites (Fig. 5C, image a) do not show ESVs, but under encystation stimuli, ESV synthesis takes place (Fig. 5C, image b). While gGlcT1 overexpression produces enlarged and aggregated ESVs (Fig. 5C, image d), knocking down (Fig. 5C, image c) of this enzyme (using anti-gGlcT1 morpholino oligonucleotide) abolishes the biogenesis of these transport vesicles. Moreover, in rescued cells (Fig. 5C, image e), the biogenesis of ESVs was restored compared to +gGlcT1-encysting cells (Fig. 5C, image d), which appear to be normal and well segregated. Based on these results and our previous published report [15], it can be postulated that ESV biogenesis is linked to gGlcT1 activity and its modulation (by overexpression and knockdown) interferes with intracellular signaling events that influence the biogenesis and transport of the ESVs.

4. DISCUSSION

We have previously reported that GlcT1 activity in *Giardia* is upregulated during encystation, and the modulation of its activity by overexpression and knockdown interferes with encystation and cyst production [15]. In this study, we show that gGlcT1 is a promiscuous enzyme that is able to utilize both UDP-Glc and UDP-Gal. To better understand the nature of this enzyme, we characterized gGlcT1 activity in live cells, cell lysate, and in crude membrane fractions. We also demonstrate that the modulation of gGlcT1 activity alters CWP expression, which implies that gGlcT1 functions as a regulator of encystation and may work in tandem with CWPs and other proteins involved during cyst formation.

Accumulated evidence suggests that GlcCer, the enzymatic product of GlcT1, participates in various cellular processes in mammals [36], plants [37], and fungi and protozoa [38]. For example, in *Cryptococcus*, GlcCer synthesis is critical for maintaining pathogenicity [39]. Likewise, GlcCer is essential for producing virulent and infective strains of *Candida albicans* [40]. In *Arabidopsis thaliana*, it has been shown that GlcCer biosynthesis is important for

regulating the morphology of Golgi complex and protein secretion [30]. In *Giardia*, GlcT1 enzymatic activity is regulated during encystation and modulation of the expression of the *gglct1* gene by overexpression and knockdown interferes with encystation and cyst production [15]. While gGlcT1 overexpression alters lipid uptake and metabolism and generates non-viable cysts [15], the pharmacological inhibition of this enzyme by PPMP increases intracellular ceramide concentration and interferes with cytokinesis, cell division, and encystation [26]. Like GlcT1, ceramide galactosyltransferase (CGalT) is also an important enzyme in higher eukaryotes and located in the lumen of the ER for GalCer synthesis [41].

Because gGlcT1 plays an important role in giardial biology, we were interested in characterizing this enzyme further. Along with UDP-Glc, we also tested other sugar nucleotides such as UDP-Gal and GDP-mannose to evaluate the substrate affinities of gGlcT1. We employed a non-radioactive NBD-ceramide as an acceptor molecule to monitor NBD-GlcCer and NBD-GalCer synthesis. NBD-ceramide was also helpful to follow the synthesis of NBD-GlcCer and NBD-GalCer by live *Giardia*. It was observed that NBD-ceramide is taken up by live *Giardia* and a significant amount of this lipid is glycosylated to NBD-GlcCer and NBD-GalCer mostly in encysting cells (Fig. 1). In fact, non-encysting trophozoites were unable to produce NBD-GlcCer and NBD-GalCer from NBD-ceramide, suggesting that this reaction is mostly induced during encystation. The synthesis of NBD-GalCer by *Giardia* raises several questions. As the gene for CGalT is not present in *Giardia* (www.GiardiaDB.org), we wondered whether the same or different enzymes were responsible for producing both NBD-GlcCer and NBD-GalCer during encystation. For this, gGlcT1-overexpressed and *gglct1* gene knockdown was used to evaluate the synthesis of these two hexosylceramides. It was observed that the overexpression and knockdown of the *gglct1* gene modulated the syntheses of both products and found that the amount of NBD-GalCer was higher than the amount of NBD-GlcCer (Fig. 1).

The observation—i.e., NBD-GalCer synthesis by live cells and its modulation by *gglct1* gene overexpression/knockdown—encouraged us to examine the activity in crude membrane fractions (15 000g pellet) (Fig. 2). Results shown that gGlcT1 enzyme was enriched on crude membrane fractions and that NBD-GalCer synthesis was higher than NBD-GlcCer. This finding indicates that the *gglct1*-encoded product may carry the activities of both transferases and that the preference of a particular substrate (i.e., UDP-Glc or UDP-Gal) depends on intracellular locations of the enzyme and availability of the ceramide molecule. Hillig et al. [28] observed that GlcT1 in cottonseed (*G. arborium*) can catalyze the formation both GlcCer and sterol glucoside. In cottonseed, this enzyme is located in the ER membranes as well as in the apoplastic side of the plasma membranes. While the apoplastic enzyme can produce both GlcCer and sterol glucoside, the ER enzyme facilitates the formation of GlcCer. This can also be true in the case of gGlcT1, which shows different rate of the synthesis of GlcCer and GalCer from respective UDP-sugars (Fig. 3). Holzl et al. [42] found that glucosyl/galactosyltransferases from bacteria *Agrobacterium tumefaciens* and *Mesorhizobium loti* display multiple substrate specificities and can transfer three successive glucosyl- and galactosyl residues to diacylglycerol. Recently it has been reported that some giardial enzymes of carbohydrate metabolic pathways are promiscuous in nature and can utilize more than one substrate [43]. For instance, UDP-glucose pyrophosphorylase in

Giardia can utilize galactose-1-phosphate. This allows the cell to bypass several steps to produce UDP-Gal from galactose-1-phosphate because the genes for galactose transferase, galactose kinase, UDP-galactose pyrophosphatase, and phosphoglucomutase are not present in this parasite [43]. [Likewise, pyridoxal phosphate-dependent alanine racemase shows promiscuity with cystathion β lyase and exhibits different K_m and K_{cat} values. It has been proposed that that overlapping patterns of promiscuity may result from sharing a common, multi-functional ancestor enzyme [44].

Since the giardial genome is highly reduced and many metabolic genes are absent [45], it is likely that *Giardia* has evolved mechanisms to use the same enzyme for different substrates to carry out various cellular reactions. In *Giardia*, it is not clear why gGlcT1 carries GalCer activity (Fig.s 1–3). [Our bioinformatic analysis clearly indicate that gGlcT1 has both CGlcT and CGalT domains (Fig. 4), suggesting that it might act as a promiscuous enzyme, however it is important identify the catalytic domains of both enzymes to support this possibility. It is also likely that, in *Giardia*, a single catalytic domain can carry out the transfer of UDP-sugars to ceramide but the binding sites of UDP-glucose and UDP-galactose are separate.]

Because encystation or gGlcT1 overexpression stimulates the synthesis of NBD-GlcCer and NBD-GalCer (Fig. 1), and CWP synthesis is induced during the conversion of trophozoites to cysts [46], we wondered whether gGlcT1 expression and CWP synthesis are interlinked and strictly regulated during encystation. We found that the synthesis of both gGlcT1 and CWP-1 are stimulated in 24-h post-induction of encysting cells (24 h.p.i.e) (Fig. 5), suggesting that the expressions of these two proteins can be regulated by the same signaling components of encystation and facilitate the process of cyst production. However, it is not known whether gGlcT1 and CWPs interact or regulation takes place at the transcription level. Co-purification of these two proteins followed by proteomic analysis should be helpful to examine this possibility in the future. It will be also interesting to test the idea of whether GlcCer and GalCer are involved in producing proinflammatory cytokines during the host-*Giardia* interactions. Recent reports suggest that small glycosphingolipids such as α . GalCer produced by intestinal microbes may activate invariant natural killer T (NKT) cells in the gut and produce pro-inflammatory cytokines [47]. Therefore, it is likely that GlcCer and GalCer produced by *Giardia* (either released or associated with broken parasitic cells) can cause inflammatory intestinal bowel-like syndrome in humans and such symptoms may persist even after the complete elimination of giardiasis.

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Abbreviations

BLAST

basic local alignment search tool

CGlcT	ceramide glucosyltransferase
CGalT	ceramide galactosyltransferase
ESV	encystation-specific vesicles
gGlcT1	giardial glucosylceramide transferase 1
GlcCer	glucosylceramide, GalCer, galactosylceramide
GDP	guanosine diphosphate
GSL	glycosphingolipid
HMM	hidden Markov model
h.p.i.e	hours post-induction of encystation
KD	knockdown
NBD-ceramide	N-[7-(4-nitrobenzo-2-oxa-1, 3-diazole)]-ceramide
PDI-2	protein-disulfide isomerase-2
TLC	thin-layer chromatography
UDP	uridine diphosphate
UDP-Glc	UDP-glucose
UDP-Gal	UDP-galactose

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Highlights

- Glucosylceramide transferase (GlcT1) catalyzes the transfer of UDP-glucose to ceramide.
- The activity of giardial GlcT1 (gGlcT1) is upregulated during encystation.
- gGlcT1 is a dual substrate enzyme and utilizes both UDP-glucose and UDP-galactose.
- The rate of galactosylceramide synthesis by gGlcT1 is higher than glucosylceramide.
- Profile hidden Markov analysis reveals that gGlcT1 contains conserved domains of ceramide glucosyltransferase (CGlcT) and ceramide galactosyltransferase (CGalT).
- gGlcT1 expression and cyst wall protein synthesis are co-regulated.

Fig. 1.

Glycosylation of NBD-ceramide by live *Giardia*. (A) Control, gGlcT1-overexpressed parasites (+gGlcT1) and *ggT1* gene knockdown (KD). Parasites were starved for 1 h at 37°C before incubating in the presence of 15 μ M NBD ceramide for 3 h as described in Materials and methods. Glycosphingolipids were extracted [15] from NBD-ceramide labeled cells and separated by TLC as also described in the methods section. (B) The fluorescent chromatograms were analyzed by densitometry and the amount of NBD-GlcCer and NBD-GalCer produced was expressed as pmol/h $\times 10^7$ cells. The bar represents the mean ($\pm$ SE) of two independent experiments (carried out in duplicate). Data analysis were performed with one-way ANOVA and Bonferroni correction comparing basal levels from encysting control cells with other experimental conditions; *** $p < 0.001$, ns: non-significant. Troph, trophozoites; encyst, encysting cells (24 h.p.i.e); KD, knockdown.

Fig. 2.

Glycosylation of NBD-ceramide by *Giardia* cell lysate, crude membrane and immunoprecipitated (IP) fractions. **(A)** Separation of gGlcT1 products. The reactions were carried out either in the presence of UDP-Glc (1 mM) or UDP-Gal (0.5 mM) and NBD-ceramide (5 μ M). The products were analyzed by TLCs. **(B)** Quantitative assessment of GlcT1 activity (crude membrane fractions, P₁₅₀₀₀) in control, gGlcT1 overexpressing (+gGlcT1), *gglcT1* knockdown (KD) and rescued cells. The reaction products were analyzed by TLC, bands were quantified by densitometry scanning, and gGlcT1 activities were expressed in the nmol of products (NBD-Glc or NBD-Gal) that formed per min per mg. protein. Bars represent the mean of GlcT1 activity ($\pm$ SE). Data analysis was performed with one-way ANOVA and Bonferroni correction comparing encysting control *Giardia* cells with other experimental conditions. **(C)** Immunoprecipitation (IP) of gGlcT1. gGlcT1 was immunoprecipitated from control and +gGlcT1 cells (described in methods) using monoclonal AU1-Sepharose (Covance). The IP fractions were incubated with NBD-ceramide (5 μ M), UDP-Glc (1mM) or UDP-Gal (50 μ M) to determine gGlcT1 activity and the products were analyzed by TLC. **(D)** Quantitation of NBD-GlcCer and NBD-GalCer in immunoprecipitation (IP) fractions. The products (NBD-GlcCer or NBD-GalCer) derived from GlcT1 reactions were analyzed by densitometry and the activity was expressed in pmol/min. The bars represent the mean of GlcT1 activity ($\pm$ SE) of two independent experiments (carried out in duplicate). Data analysis was performed with t-test comparing encysting *Giardia* cells (control) with +gGlcT1 cells; * p < 0.05, ** p < 0.01; *** p < 0.001. troph, trophozoites; encyst, encysting cells (24 h.p.i.e); KD, knockdown.

Fig. 3.

The kinetic analysis and substrate competition. **(A)** UDP-Glc and UDP-Gal-dependent activity of gGlcT1. The reaction was carried by incubating membrane fraction (15000×g pellet) with NBD-ceramide (5 μ M) and various concentrations (0-0.5 mM) of UDP-Glc (●) or UDP-Gal (○) followed by analyzing the products (NBD-GlcCer and NBD-GalCer) by TLCs. Curves generated from the reactions were adjusted to the Michaelis–Menten model and the kinetic parameters were calculated accordingly. The specific activity was defined as the nmol of products formed/min/mg protein. Error bars represent the mean ($\pm$ SE) of three independent experiments carried out in duplicate. **(B)** Dual substrate specificity of gGlcT1. The formation of NBD-GlcCer and NBD-GalCer was measured in the presence of UDP-Glc (1 mM) with varying concentrations (0.05-0.5 mM) of UDP-Gal. **(C)** The synthesis of NBD-GlcCer and NBD-GalCer in the presence of UDP-Gal (0.05 mM) with varying concentration of UDP-Glc (0.05-1.0 mM). In both cases (B and C), the amount of NBD-ceramide (5 μ M) was unchanged. Bars represent the mean of GlcT1 activity ($\pm$ SE) of three independent experiments carried out in duplicate. Statistical analyses were performed with one-way ANOVA and Bonferroni correction comparing control with other experimental conditions; * $p < 0.05$; *** $p < 0.001$; ns, non-significant.

Fig 4.

Schematic representation of gGlcT1 with multiple sequence alignments to CGlT and CGalT. Conserved residues corresponding to NRD 2-L and NRD 2-S domains [31] are shown above and were identified by an aligning CGlT (A8BXJ1), amino acid sequence to CGlT sequences found in the following taxa: *Physcomitrella patens subsp. patens* (est Ext gwp gw1.C 1940082), *Ricinus communis* (29912.m005431), *Homo sapiens* (Q16739), *Mus musculus* (O88693), *Komagataella pastoris* (strain GS115/ATCC 20864 (C4R4B3), *Xenopus laevis* (Q8AY29), *Caenorhabditis elegans* (Q21054), *Synechocystis sp.* (strain PCC 6803/Kazusa) (P74046), *Magnaporthe oryzae* (strain 70-15/ATCC MYA-4617/FGSC 8958) (G4MS28). A conserved region in CGlT1 (242-267) shows homology with vertebrate CGalT sequences in an alignment with the following taxa: *Bos Taurus* (A4IFB0), *Homo sapiens* (Q16880), *Mus musculus* (Q64676), *Rattus norvegicus* (Q09426), *Gallus gallus* (Q98TB5), *Canis lupus familiaris* (E2RA42), *Felis catus* (M3WV11), *Xenopus tropicalis* (F6W899). Like CGalT, CGlT has a predicted C-terminal transmembrane domain and a conserved ER retrieval sequence.

Fig. 5.

Modulation of gGlcT1 activity influences CWP synthesis and ESV biogenesis.

(A) Immunoblot analysis using gGlcT1, CWP and PDI2 antibodies. (B) Intensity levels of GlcT1 and CWP1 relative to PDI2 intensity values. (C) ESV biogenesis was monitored in control, +gGlcT1, *gglct1* gene knockdown (KD) and rescued cells (rescue). Nuclear peripheral membranes were stained with wheat germ agglutinin (WGA, green) and ESVs were labeled with CWP antibody conjugated to Alexa Fluor 594, red). Scale bar, 5 μ m. All of the data analysis was performed with one-way ANOVA and Bonferroni correction comparing control encysting cells. *** $p < 0.001$; ns, non-significant. troph, trophozoites; encyst, encysting cells (24 h.p.i.e); KD, knockdown; +GlcT1, gGlcT1 overexpressed; GlcT1, glucosylceramide transferase; CWP, cyst-wall protein; PDI2, protein disulfide isomerase-2.