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Identification of a family of four UDP-polypeptide N-acetylgalactosaminyl transferases in *Cryptosporidium* species

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Abstract

Although mucin-type O-glycans are critical for *Cryptosporidium* infection, the enzymes catalyzing their synthesis have not been studied. Here, we report four UDP N-acetyl- α -D-galactosamine:polypeptide N-acetylgalactosaminyl transferases (ppGalNAc-Ts) from the genomes of *C. parvum*, *C. hominis* and *C. muris*. All are Type II membrane proteins which include a cytoplasmic tail, a transmembrane domain, a stem region, a glycosyltransferase family 2 domain and a C-terminal ricin B lectin domain. All are expressed during *C. parvum* infection *in vitro*, with *Cp*-ppGalNAc-T1 and -T4 expressed at 24 h and *Cp*-ppGalNAc-T2 and -T3 at 48 and 72 h post-infection, suggesting that their expression may be developmentally regulated. *C. parvum* sporozoite lysates display ppGalNAc-T enzymatic activity against non-glycosylated and pre-glycosylated peptides suggesting that they contain enzymes capable of glycosylating both types of substrates. The importance of mucin-type O-glycans in *Cryptosporidium*-host cell interactions raises the possibility that *Cp*-ppGalNAc-Ts may serve as targets for intervention in cryptosporidiosis.

Keywords

O-glycosylation; Mucin; Mucin-like glycoprotein; *Cryptosporidium*; UDP GalNAc:polypeptide; N-acetylgalactosaminyl transferase

Cryptosporidium species are water-borne apicomplexan parasites, which cause gastrointestinal disease worldwide [1] which can be particularly devastating in immunocompromised individuals, such as untreated AIDS patients or malnourished children in developing countries. Nitazoxanide, the only drug approved for treatment of

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.molbiopara.2013.08.002>.

cryptosporidiosis in immunocompetent individuals by the US Food and Drug Administration is not effective in the immunocompromised.

Mucin-type O-glycosylation is a critical post-translational modification in *Cryptosporidium* species. Unlike other apicomplexans, *Cryptosporidium* employs O-glycosylated mucin-like glycoproteins to attach to and infect intestinal epithelial cells [2]. In addition, the *Cryptosporidium* genomes contain several additional genes which are predicted to encode mucins or mucin-like glycoproteins [3,4].

Previously, we showed that the O-glycans on mucin-like glycoproteins gp900 and gp40 display terminal O-linked glycans GalNAc α 1-Ser/Thr and/or Gal β 1-3GalNAc α 1-Ser/Thr, the Tn and T antigens respectively [5]. Lectins specific for these O-glycans block attachment and irreversibly inhibit infection of host cells *in vitro* [5,6]. A monoclonal antibody 4E9 directed against these O-glycans inhibits infection *in vitro* [5] and *in vivo* (Ward H., unpublished). Taken together, these data strongly suggest that mucin-type O-glycans are crucial for *Cryptosporidium* species infection. However, the enzymes that catalyze the synthesis of O-glycans in *Cryptosporidium* have not been studied.

Mucin type O-glycosylation is initiated by UDP *N*-acetyl- α -D-galactosamine:polypeptide *N*-acetylgalactosaminyl transferases (ppGalNAc-Ts, EC 2.4.1.41), which transfer α GalNAc from UDP-GalNAc to the hydroxyl group of serine or threonine residues on acceptor proteins [7,8]. Different isoforms of ppGalNAc-Ts display unique, as well as overlapping, substrate specificities with some functioning as “initiating” enzymes that transfer GalNAc to unmodified peptide substrates, whereas others function as “follow-up” enzymes that transfer GalNAc to peptides pre-glycosylated with α GalNAc [7,8]. ppGalNAcTs are evolutionarily conserved and were identified in a wide variety of organisms ranging from mammals to protozoa. Currently, there are 94 distinct ppGalNAc-T gene families [7]. The human genome encodes 20 ppGalNAc-Ts, many of which were functionally characterized [8]. Among protozoa, ppGalNAcTs have thus far only been reported in *Toxoplasma gondii* [9–11].

Mining of the *C. parvum* genome in CryptoDB (www.cryptodb.org) [12] revealed four genes (gene IDs cgd7_4160, cgd6_1960, cgd5_690 and cgd7_1310) encoding putative ppGalNAc-Ts, which we named *C. parvum* (*Cp*)-ppGalNAc-T1, T2, T3 and T4, respectively. Sequences encoding all four genes were PCR-amplified from *C. parvum* (Iowa isolate, obtained from Bunch Grass Farms, ID) genomic DNA using specific primers (Table S1), cloned into the TOPO pCR2.1 vector (Invitrogen, Carlsbad, CA) and their sequences verified. Orthologs of *Cp*-ppGalNAc-T1, T2, T3 and T4 were identified in the genomes of *C. hominis* (gene IDs Chro. 70461, Chro. 60231, Chro. 50322 and Chro. 70457 respectively) and *C. muris* (gene IDs CMU 014290, CMU 007930, CMU 041230 and CMU 014290 respectively) in CryptoDB. The *Ch*-ppGalNAc-T1 and *Cp* and *Ch*-ppGalNAc-T4 genes were incompletely annotated in CryptoDB and lacked the 5' regions encoding putative transmembrane domains and cytoplasmic tails for *Cp* and *Ch*-ppGalNAc-T4 and the 3' region for *Ch*-ppGalNAc-T1. These regions were identified from their respective contigs using the correctly annotated *C. muris* (for *Cp* and *Ch*-ppGalNAc-T4) and *C. parvum* (for *Ch*-ppGalNAc-T1) genes as templates. The sequences of these regions were confirmed by cloning (into the TOPO pCR2.1 vector) and sequencing them from *C. hominis* genomic DNA (TU502 isolate, obtained from Dr. Saul Tzipori, Tufts University Cummings School of Veterinary Medicine, N. Grafton, MA) for *Ch*-ppGalNAc-T1 and *C. parvum* genomic DNA for *Cp*-ppGalNAc-T4.

Analysis of the completely annotated sequences from all three species revealed that, like most other *Cryptosporidium* genes, those encoding ppGalNAcs-T1, 2 and 3 do not contain

introns. However, ppGalNAc-T4 from all three species contains a short intron at their 5' ends. Analysis of the deduced amino sequences indicate that all four ppGalNAc-Ts from each *Cryptosporidium* species are Type II membrane proteins which display the characteristic domain structure of ppGalNAc-Ts, including a short amino-terminal cytoplasmic tail, one or more N-terminal transmembrane domains, a stem region, a glycosyltransferase family 2 domain and a ricin B lectin domain (schematics depicting the *Cp*-ppGalNAc-Ts domain structures are shown in Fig. 1A). The first exon of the ppGalNAc-T4s encodes the cytoplasmic tail and first transmembrane domain; the second exon encodes the transmembrane domain, stem region, glycosyltransferase family 2 domain and ricin B lectin domain.

Interestingly, ppGalNAc-T2 and -T4 from all three *Cryptosporidium* species have three transmembrane helices, as compared to most other known ppGalNAc-Ts, which have a single transmembrane domain. The GT-A motif of all *Cryptosporidium* ppGalNAc-T catalytic domains contain the conserved, invariant DXH Mn²⁺ cation binding motif (not shown). Similarly, they all contain the conserved DXXXXXWGXENXE sequence containing invariant D, E and E residues in the Gal/GalNAc-T motif and display the typical α , β and γ repeats in the ricin B lectin domain (not shown).

The deduced amino acid sequences of *Cp*-ppGalNAc-T1-T4 were aligned with the *C. hominis* and *C. muris* orthologs and with human homologs (Tables S2 and S3). Within each *Cryptosporidium* species, identity among the four different ppGalNAc-Ts ranged from 12 to 36% for *C. parvum*, 10 to 37% for *C. hominis* and 13 to 44% for *C. muris*. However, among these three, the ppGalNAc-T orthologs were more similar to each other (63–99% identity for *Cp*-ppGalNAc-T1, 44–98% for *Cp*-ppGalNAc-T2, 51–98% for *Cp*-ppGalNAc-T3 and 57–97% for *Cp*-ppGalNAc-T4) than to the other *Cp*-ppGalNAc-T isoforms within the same species. Identities of *Cryptosporidium* species ppGalNAc-Ts with human homologs ranged from 12 to 27% (Table S3).

We searched EupathDB (<http://eupathdb.org/eupathdb/>) [13] for ppGalNAc-Ts in the genomes of other apicomplexan parasites using the *Cp*-ppGalNAc-T sequences for BLAST comparison. In addition to the previously identified ppGalNAc-Ts from *Toxoplasma gondii* [9], we identified ppGalNAc-Ts containing the characteristic glycosyltransferase family 2 and ricin B lectin domains in *Neospora caninum* (gene IDs NCLIV_0272, NCLIV_0277, NCLIV_010830, NCLIV_067480 and NCLIV_029020) and *Eimeria tenella* (gene IDs ETH_00002780, ETH_00005370, ETH_00029115). Interestingly, among the apicomplexan genomes sequenced to date, ppGalNAc-Ts were only identified in *Cryptosporidium*, *Toxoplasma*, *Neospora* and *Eimeria* species, all of which form environmentally-resistant oocysts and invade the gastrointestinal tract of humans and other animals. However, these enzymes were not identified in the genomes of other apicomplexans including *Plasmodium*, *Babesia* or *Theileria*, all of which are vector-borne hemoparasites. The findings that O-glycans are present in *Cryptosporidium* [5,6], *Toxoplasma* [14] and *Eimeria* [15], but not in *Plasmodium*, *Babesia* or *Theileria* species are consistent with the lack of ppGalNAc-Ts in the latter three apicomplexans.

The presence of expressed sequence tags (in CryptoDB) for each of the *Cp*-ppGalNAc-Ts indicated that all four are expressed at the RNA level. To confirm this and to determine their transcriptional profile during infection *in vitro*, we performed reverse transcription (RT) PCR on RNA extracted from *C. parvum*-infected-HCT-8 cells at 24, 48 and 72 h post infection using primers specific for each (Table S1). All four *Cp*-ppGalNAc-Ts were expressed, though at different times during intracellular development. *Cp*-ppGalNAc-T-1 and 4 were expressed at 24 h post-infection, whereas *Cp*-ppGalNAc-T2 and T3 were expressed at 48 and 72 h post-infection (Fig. 1B).

Recent data on the transcriptome of intracellular stages of *C. parvum* (assessed by high throughput real time PCR at varying time points ranging from 2 to 72 h post-infection) [16] indicated that all four ppGalNAcTs are expressed throughout infection *in vitro*. However, in this study relative expression levels of all four ppGalNAcTs varied considerably at different time points during infection as well as among four different time course experiments. The median time of maximal expression (normalized to that of the 18S rRNA gene) as reported in CryptoDB was 36, 12, 36 and 24 h post-infection for CpppGalNAc-T-1, 2, 3 and 4 respectively. Another recent study which analyzed the *C. parvum* transcriptome in oocysts using microarray technology [17] indicated that *Cp*-ppGalNAc-T1 is not expressed in this stage and that *Cp*-ppGalNAcs-T3 and T4 are in the lowest quartile of those genes expressed in oocysts. Information on expression of *Cp*-ppGalNAc-T4 in oocysts was not provided.

Because *C. parvum* infection *in vitro* is asynchronous, it is not possible to assign expression to a particular developmental stage at a given time point post-infection. However, O-glycans have been identified in multiple developmental stages suggesting that differential expression may indicate developmental regulation of *Cp*-ppGalNAc-Ts. Different ppGalNAc-T isoforms in higher eukaryotes, including humans, are also differentially expressed in specific cells or tissues [7]. Clearly, additional studies are required to determine whether expression of *Cp*-ppGalNAc-Ts is developmentally regulated.

Since *Cp*-ppGalNAc-Ts are expressed at the RNA level, we then determined if ppGalNAc-T activity could be detected in *C. parvum* sporozoite lysates. Excysted sporozoites were lysed in 0.5 ml of 20 mM Tris-HCl buffer pH 7.2 containing 1% Triton X-100, 135 mM NaCl, 10 mM MnCl₂ containing a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, IL), and incubated on ice for 2 h. The supernatant was collected by centrifugation at 9100 × *g* for 25 min and designated “sporozoite lysate”. The protein content of lysates was measured using a Pierce micro BCA kit (Thermo Scientific, Rockford, IL).

Two different assays were used to measure enzyme activity in sporozoite lysates. In the first, activity was measured by quantifying transfer of [¹⁴C]-labeled GalNAc to synthetic biotinylated peptides and glycopeptides as described [11]. Because the endogenous *C. parvum* peptide/glycopeptide acceptors are unknown, we used “standard” peptides/glycopeptides derived from known mucins that have been used to evaluate other ppGalNAc-Ts. As shown in Table 1, *C. parvum* sporozoite lysates did indeed exhibit ppGalNAc-T activity. Equivalent activity was detected using both peptide (EA2, N or C-terminally biotinylated) and glycopeptide (EA2-2, which is pre-glycosylated with αGalNAc on the second {Thr} residue) acceptors, suggesting the presence of both “initiating” and “follow up” enzyme activity in *C. parvum*. However, EA2-7 which is pre-glycosylated with αGalNAc on the 7th (Thr) residue was not glycosylated suggesting that *Cp*-ppGalNAcTs may not display specificity for this substrate. This substrate specificity is different from that of recombinant *T. gondii* enzymes *Tg*-ppGalNAc-T1, which did not glycosylate EA2, EA2-2 or EA2-7 [10] and *Tg*-ppGalNAc-T3 which did not glycosylate EA2 or EA2-2 but did glycosylate EA2-7 [11] using the same assay. Non-glycosylated MUC5AC (N or C-terminally biotinylated) was not glycosylated whereas MU5AC 3, 13 which is pre-glycosylated on the 3rd and 13th residue was glycosylated by the *C. parvum* sporozoite lysate as well as by recombinant *Tg*-ppGalNAc-T1 and 3 [10,11]. Interestingly, the non-glycosylated GRA2 peptide derived from the *T. gondii* GRA2 glycoprotein [18] was robustly glycosylated by the *C. parvum* sporozoite lysate but not by *Tg*-ppGalNAc-T1 and 3 [10,11].

The second assay is an enzyme-linked lectin assay (ELLA), which was developed for high throughput screening of inhibitors of mammalian ppGalNAc-Ts using EA2 as an acceptor peptide [19]. Briefly, increasing concentrations of biotinylated EA2 acceptor peptide and the

sugar nucleotide donor UDP-GalNAc were incubated with *C. parvum* sporozoite lysate and then added to 96-well neutravidin-coated plates. The immobilized glycopeptide product was detected using the α GalNAc-specific lectin, *Helix pomatia* agglutinin (HPA) conjugated to horseradish peroxidase followed by addition of a chromogenic substrate. As seen in Fig. 2, GalNAc transfer increased with increasing concentrations of the EA2 peptide. Maximum activity was obtained at 100 μ M EA2, after which activity plateaued. These data confirm the presence of initiating ppGalNAc-T enzymatic activity with the EA2 peptide in *C. parvum* sporozoites. However, the specific *Cp*-ppGalNAc-Ts responsible for this activity and their substrate specificities remain to be determined.

In conclusion, in this study, we identified, cloned and characterized *in silico* a family of four *C. parvum* ppGalNAc-Ts and identified orthologs of each of them in *C. hominis* and *C. muris* as well as homologs in the related apicomplexan parasites *N. caninum* and *E. tenella*. We showed that all four *Cp*-ppGalNAc-Ts are differentially expressed during infection *in vitro* and quantified initiating and follow up ppGalNAc-T enzymatic activity in *C. parvum*. Current efforts are directed at expressing enzymatically active recombinant forms of all four *Cp*-ppGalNAc-Ts to determine which of them display initiating and/or follow up activity, to identify the native *C. parvum* peptide and glycopeptide substrates which are O-glycosylated by these enzymes, to determine whether they are developmentally regulated and to elucidate their role in *C. parvum*-host cell interactions.

Because O-glycans are crucial for *C. parvum* infection of host cells, *Cp*-ppGalNAc-Ts may serve as therapeutic targets for drug development against cryptosporidiosis. If so, it may be possible to design specific inhibitors that target parasite, but not host, enzymes. Characterization of the structure and function of these enzymes is essential in order to investigate this possibility.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

(A) Domain structure of *Cp*-ppGalNAc-Ts. The domain structure of *Cp*-ppGalNAc-Ts was predicted using the InterProScan server (<http://www.ebi.ac.uk/Tools/pfa/iprscan/>). The presence of a transmembrane domain (TMD) was predicted using InterProScan and TMHMM Server v. 2.0 (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>). CT, cytoplasmic tail; SR, stem region; CD, catalytic domain; GT-A, glycosyl transferase A domain; RBLD, ricin B lectin domain. The GT-A domain contains a Gal/GalNAc motif, and the RBLD contains a β Trefoil fold. B. Time course of expression of *Cp*-ppGalNAc-Ts in *C. parvum*-infected HCT-8 cells. HCT-8 cells were infected with *C. parvum* oocysts for 24, 48 and 72 h. RT-PCR was performed on total RNA extracted from uninfected and *C. parvum*-infected HCT-8 cells using the primers listed in Table S1 as described [20]. RNA from uninfected cells was used as a negative control. RT-PCR reactions without added reverse transcriptase were performed to exclude the presence of contaminating DNA. Genomic DNA was used as a positive control and the 18S rRNA gene was used as an internal control.

Fig. 2. ppGalNAc-T enzymatic activity in *C. parvum* lysates. Enzyme activity in *C. parvum* sporozoite lysates was measured with increasing concentrations of the EA2 peptide as an acceptor substrate using an enzyme linked lectin assay (ELLA) as described [19]. Assays were performed in duplicate and the data shown are the mean $\pm$ SE of two independent assays.

Table 1

ppGalNAc-T activity in *C. parvum* sporozoite lysates.

Acceptor name	Acceptor sequence	Activity
1. Biotin-EA2	Biotin-PTTDSTTPAPTTK	198.0
2. Biotin-EA2-2	Biotin-P <u>TTD</u> STTPAPTTK	179.1
3. Biotin-EA2-7	Biotin-PTTDST <u>TP</u> APTTK	13.5
4. Biotin-MUC5AC	Biotin-GTTPSPVPTTSTTSAP	18.8
5. Biotin-MUC5AC-3,13	Biotin-GT <u>TP</u> SPVPTTST <u>TS</u> AP	93.3
6. MUC5AC-biotin	GTTPSPVPTTSTTSAPK-biotin	0
7. EA2-biotin	PTTDSTTPAPTTK-biotin	177.5
8. GRA2-biotin	VESDRSTTTTQAPK-biotin	273.8

The transfer of [^{14}C] GalNAc to biotinylated peptides (Alpha Diagnostics International, San Antonio, TX) by ppGalNAc-Ts in sporozoite lysates was quantified, as described previously [11]. The pre-glycosylated peptides (residues in bold and underlined) which were pre-glycosylated with α GalNAc were obtained from Dr. Carolyn Bertozzi (National Institutes of Health, Bethesda, MD). Enzyme activity was expressed as nmoles of UDP-GalNAc transferred per mg of protein per minute (nmol GalNAc/mg/min).