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Stable expression of *Cryptosporidium parvum* glycoprotein gp40/15 in *Toxoplasma gondii*

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Abstract

Cryptosporidium is a cause of diarrheal disease worldwide. Parasite glycoproteins involved in invasion of *Cryptosporidium* into host cells have been investigated as possible targets for effective interventions against this parasite. One of these, *Cpgp40/15*, is expressed as a precursor protein that is cleaved by a parasite-derived furin-like protease activity into gp15, a glycosphosphatidyl inositol anchored surface protein, and gp40, that associates with gp15 and binds to host cells. Investigation of the functions of these glycoproteins requires an expression system that can produce similar glycosylation patterns to the native antigens. Previous work demonstrated that *Cpgp40/15* transiently expressed in *T. gondii* was appropriately localized and glycosylated. In this study, *T. gondii* stable transfectants expressing gp40/15, gp15, gp40 and hemagglutinin (HA) tagged gp40 were generated. *T. gondii* recombinant gp40HA and gp40/15 (*recTgpgp40HA* and *recTgpgp40/15*) were isolated from infected cells by HA affinity chromatography and *Helix pomatia* lectin affinity chromatography, respectively. Mass spectrometry confirmed that *recTgpgp40-HA* and native *Cpgp40* were similarly glycosylated. Like native *Cpgp40/15*, *recTgpgp40/15* could be cleaved into the gp40 and gp15 products by human furin or by a furin-like protease activity in *T. gondii* tachyzoite lysates. However, processing was inefficient in intact tachyzoites. Unlike the N-terminus of native *Cpgp40/15*, which appears to be processed following signal peptide cleavage, the N-terminus of *recTgpgp40/15* began at the predicted signal sequence cleavage site, 11 amino acids upstream of the N-terminus of native *Cpgp40*. The ability to express and isolate appropriately glycosylated *Cryptosporidium* glycoproteins will enable further investigations into host-parasite interactions of this important pathogen.

Keywords

Cryptosporidium; *Toxoplasma*; mucin-like glycoproteins; proteolytic processing

Introduction

Cryptosporidium is a ubiquitous waterborne pathogen of worldwide distribution [1]. The parasite is endemic in developing countries due to the lack of clean water sources, and is also

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frequently the cause of waterborne outbreaks of diarrheal disease in industrialized countries due to contamination of drinking and recreational water [2,3]. The disease, while self-limiting in immunocompetent hosts, follows a severe and sometimes fatal course in immunocompromised hosts such as AIDS patients [4] or malnourished children [5]. To date there is no vaccine to prevent cryptosporidiosis, and there is no consistently effective treatment. Nitazoxanide, which has been approved for treatment of cryptosporidiosis, has been reported to reduce duration of diarrhea and oocyst shedding but is not effective in AIDS patients [6]. Thus, there remains a need for more effective therapies.

The life-cycle of *Cryptosporidium* is confined to the host gut epithelium where the parasite undergoes asexual and sexual reproduction before producing oocysts to be released into the environment [7]. Replication of life cycle stages occurs within a parasitophorous vacuole that is uniquely located under the cell membrane, but separated from the cell cytoplasm. Although the specific molecular processes for *Cryptosporidium* host cell invasion are not well understood, the parasite appears to follow the paradigm described for other apicomplexans [8]. The apical region of the invasive zoite stages contains a collection of unique secretory organelles (the apical complex), that, upon attachment to the host cell, releases both soluble and membrane bound proteins. These proteins, as well as proteins on the surface of the zoite, facilitate attachment to the cell surface, gliding motility, and subsequent invasion and intracellular development of the parasite. However, unlike those of other apicomplexans, most of the *Cryptosporidium* surface and apical complex antigens that have been characterized are glycoproteins [9–14]. Two of these, gp900 [10] and gp40/15 [11,13,14], are mucin-like glycoproteins, proteins with high percentages of serine and threonine residues that are sites for O-linked glycosylation. Recently, the *Cryptosporidium* genome projects identified 31 putative mucin genes [15,16], suggestive of an essential role for this class of glycoprotein in *Cryptosporidium* host-parasite interactions.

Unfortunately, investigation of the molecules involved in cryptosporidial host-parasite interactions and identification of the role they play remains extraordinarily difficult [17]. One primary obstacle is the inability to genetically manipulate *Cryptosporidium*. Additionally, the complete lifecycle cannot be propagated in vitro [7]. Since the zoonotic species, *C. parvum*, is the most easily propagated in calves, research is done primarily with these parasites, despite the observation that *C. hominis* is the species responsible for most human infections [2,18]. Adding to the difficulties, the mucin-like glycoproteins that *Cryptosporidium* may rely on for attachment and invasion display an unusual “primitive” pattern of O-glycosylation with terminal α -N-acetyl-D-galactosamine (GalNAc) and Gal β 1-3GalNAc (T and Tn antigen) determinants [19] which are normally cryptic on mammalian cells due to sialylation [20]. An ideal expression system for cryptosporidial proteins would have to produce similar glycosylation patterns.

The mucin-like glycoproteins, gp40 and gp15, are among the best characterized *Cryptosporidium* antigens [11,13,14,19]. These antigens are expressed as a precursor protein (gp40/15), that is processed into gp40 and gp15 glycoproteins by a furin-like subtilase activity [21]. gp15 is anchored in the sporozoite pellicle via a glycosylphosphatidyl inositol (GPI) anchor [22], and gp40, which contains epitopes that react with an unknown host cell receptor [11], associates with gp15 on the sporozoite surface (O'Connor et. al, in preparation). Several studies have observed a high degree of polymorphism in the gp40/15 sequence among *C. hominis* isolates, all of which are highly divergent from the *C. parvum* gp40/15 sequence [13,18,23, 24]. Since gp40 is thought to be involved in attachment of sporozoites to host epithelial cells [11], polymorphisms in the gp40 sequence could translate into differences in host cell receptor recognition. Alternatively, gp40 polymorphisms may reflect selective immune pressure. Since isolation of native antigens from *C. hominis* isolates is not feasible, investigation of these

hypotheses requires the ability to express recombinant glycoproteins that mimic native antigens well enough to act as surrogate antigens in receptor-based and immunological studies.

Previous studies in our laboratory showed that the gp40 and gp15 glycoproteins could be transiently expressed in the related apicomplexan, *Toxoplasma gondii* [25]. These *T. gondii* recombinant proteins appeared to be appropriately localized and displayed glycotopes found on the native antigen, although processing was incomplete. In this report, we describe the development of stably transfected *T. gondii* lines expressing gp40 and gp15 glycoproteins. These transfected lines produce sufficient amounts of recombinant glycoproteins for purification and analysis, further supporting the feasibility of this system for generating recombinant mimics of *Cryptosporidium* mucin antigens for functional studies.

Materials and Methods

Parasites

C. parvum Iowa isolate oocysts were purchased from Bunch Grass Farm (Deary, ID). *C. parvum* parasite lysates were generated as described previously [19]. For collection of “shed” or exocytosed proteins, hypochlorite-treated oocysts were excysted in PBS at 37°C for 1 hour, the supernatant collected and protease inhibitors (Protease Inhibitor Cocktail Set III, Calbiochem, LaJolla, CA) added. The shed proteins were subjected to ultrafiltration through a 100,000 molecular weight cut-off (MWCO) Microcon concentrator (Millipore, Billerica, MA) to separate gp40 from gp900. The gp40 in the flow through was concentrated in a 30,000 MWCO Microcon concentrator. *T. gondii* RH strain parasites lacking the hypoxanthine-xanthine-guanine phosphoribosyl transferase (Δ HXGPRT) gene were obtained from Dr. David Roos, University of Pennsylvania [26] and maintained in human foreskin fibroblast (HFF) cells.

Antibodies and Lectins

4E9 is an IgM monoclonal antibody (mAb) directed against a carbohydrate epitope on the *Cryptosporidium* mucin antigens gp40 and gp900 [19]. Rabbit antisera against *E. coli* recombinant gp40 and gp15 were generated as described (O'Connor et. al in preparation). These antisera reacted specifically with the corresponding native antigen from *C. parvum* lysate in Western blots (See results). The α -N-acetyl-D-galactosamine (GalNAc)-specific lectin *Helix pomatia* agglutinin (HPA) was obtained from EY Laboratories (San Mateo, CA). Anti-p30 (SAG1) polyclonal rabbit antiserum was obtained from Dr. Lloyd Kasper, Dartmouth University.

Plasmids

The coding sequences for gp40/15, gp40 and gp15, including the gp40/15 signal sequence, were cloned into the *T. gondii* expression vector pHLEM under the control of the GRA1 promoter and upstream of the GRA2 3'UTR. This vector contains the *T. gondii* HXGPRT gene under the control of the *T. gondii* dihydrofolate reductase gene regulatory elements for the generation of stable transfectants and was obtained from Dr. David Roos, University of Pennsylvania [26]. Table 1 lists the primers used for cloning. The forward primer incorporates an NsiI cloning site that provides the ATG start codon and results in an additional histidine residue in the signal sequence (Table 1; Signal Nsi F). The reverse primers contain a PacI cloning site that provides the stop codon (Table 1; gp40 Pac R, gp40HA Pac R and gp15 Pac R). The gp40 insert included sequence up to amino acid R²¹⁹. The gp40/15 and gp40 inserts were amplified from *C. parvum* genomic DNA, and the gp15 from the pGRAgp15 vector generated previously [25]. Since the gp40/15 sequence contains an NsiI site at base pair 739, the gp40/15 and the gp15 inserts were amplified in two pieces using primers encompassing the HindIII site in gp40/15 (HindIII F and R) and the AluI site in gp15 (Alu F and R). The two

fragments and the vector were each cut with the appropriate restriction enzymes and then ligated together. To generate hemagglutinin (HA) tagged gp40, *C. parvum* genomic DNA was amplified with Signal Nsi F and a reverse primer that incorporated the 27 nucleotide coding sequence for HA and the PacI cloning site (gp40HA Pac R). All inserts were sequenced by the dye-terminator method on a Perkin-Elmer ABI 377 sequencer at the Tufts University School of Medicine Core Facility. Plasmids containing the correct sequences were purified by cesium chloride density gradient centrifugation or by using a Qiagen Megaprep Kit (Qiagen, Valencia, CA). Plasmids were designated pHLEMgp40/15; pHLEMgp40; pHLEMgp40HA; and pHLEMgp15.

Transfections

T. gondii tachyzoites were transfected with 100–150 g of plasmid as previously described [27] using a Biorad Gene Pulser II electroporator (Bio-Rad, Hercules, CA) at settings of 1.9 kV and 50 F capacitance. After a 10-minute incubation at room temperature, the parasites were added to HFF monolayers in 25 mm² flasks and transfectants selected in Dulbecco's minimum essential medium (DMEM) containing 10% fetal calf serum, 25 mM HEPES, 2X L-glutamine, 1X penicillin/streptomycin, 50g/ml xanthine and 25 g/ml of mycophenolic acid (selection medium) [26]. After 2 to 3 passages in selection medium, the transfected parasites were cloned by limiting dilution in 96 well plates. Positive clones were identified by immunofluorescence assay (IFA). Stably transfected clonal parasite lines were designated Tggp40/15, Tggp40, Tggp40-HA and Tggp15. The *C. parvum* proteins expressed by the stable transfectants were designated recTggp40/15, recTggp40, recTggp40-HA, and recTggp15.

Immunoassays

IFAs were performed on *T. gondii* intracellular stages as described [28]. Primary antibodies were detected with Alexa Fluor conjugated secondary antibodies as appropriate (Molecular Probes, Eugene, Oregon). The slides were mounted with Vectashield mounting medium containing 4',6'-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA), and examined by differential interference contrast (DIC) and fluorescence microscopy using a Zeiss AxioImager Z.1 microscope (Carl Zeiss Microscopy, Jena, Germany). Images were captured with an IEEE1394 digital CCD camera (Hamamatsu, Hamamatsu-City, Japan) and co-localization of the fluorescent labels was done using Volocity software (Improvision Inc., Lexington, MA).

For Western blot analysis, infected monolayers were scraped from the flasks, pelleted by centrifugation at 400 x g for 15 minutes and lysed in PBS, pH 7.0, containing 1% octyl glucoside (OGS) and protease inhibitor cocktail (lysis buffer). After incubation for 20 minutes on ice, the supernatants were collected by centrifugation at 16,000 x g for 30 minutes, and separated by SDS-PAGE. Proteins were transferred to PVDF membranes and probed for antibody and lectin reactivity as described previously [19].

Affinity purifications

For purification of recTggp40-HA, HFF cells were infected with Tggp40-HA and cells lysed 24 hours later in lysis buffer. After a 20 minute incubation on ice, the lysate was centrifuged (16,000 g, 10 minutes, 4°C) and the supernatant combined with anti-HA agarose (Vector Labs). The lysate and matrix were rotated together at 4°C for 2 hours, then the matrix was washed extensively with PBS-0.1% OGS. HA-tagged gp40 was eluted specifically with 1 mg/ml of HA peptide (YPYDVPDYA, synthesized by Tufts Protein Synthesis Core) in PBS, 0.1% OGS, and protease inhibitors, at 37°C. The eluted protein was concentrated and the HA peptide removed by ultrafiltration in a 30,000 MWCO Microcon concentrator.

For purification of recTgpp40/15, lysates of Tgpp40/15-infected HFF cells were prepared as described above and incubated with HPA-agarose (E-Y Laboratories, San Mateo, California) for 2 hours at 4°C. The matrix was washed with PBS-0.1% OGS, and the protein eluted with 0.25M GalNAc in PBS-0.1% OGS. The protein was concentrated and GalNAc removed by ultrafiltration in a 30,000 MWCO Microcon concentrator.

Affinity purifications were evaluated by western blot using rabbit anti-gp40 and anti-gp15 sera, mAb 4E9, and biotinylated HPA lectin (E-Y laboratories) and protein purity assessed by silver stain (Biorad, Hercules, CA) or Sypro Ruby protein gel stain (Invitrogen, Eugene, OR). Sypro Ruby red stained bands were detected on a Typhoon 8600 scanner (GE Healthcare Bio-Sciences, Piscataway, NJ) using the 610BP30 laser. To identify protein bands for liquid chromatography/mass spectrometry (LC/MS) and N-terminal sequencing, SDS-PAGE gels and western blots were stained with 1% Coomassie Brilliant Blue R-250 (USB, Cleveland, OH) in water.

Analysis of glycosylation of recTgpp40-HA

HA-affinity purified recTgpp40-HA and, for comparison, gp40 isolated from shed proteins from 10⁸ *C. parvum* oocysts were resolved on SDS-PAGE gels, and the Coomassie-stained bands excised and submitted to the Columbia University Mass Spectrometry Core Facility. Proteins were reduced, alkylated and subjected to tryptic digestion in gel as previously described [29]. The mixtures of tryptic peptides were separated on a C18PepMap column (Dionex, Marlton, NJ), using an Ultimate nano-Ic system connected directly to the nanospray source of a Micromass quadrupole-time of flight (Q-TOF) mass spectrometer. The ions produced were analyzed by a Q-TOF mass spectrometer (Micromass, Manchester, UK).

Proteolytic processing of recTgpp40/15 with furin

Tgpp40/15 tachyzoites were lysed in 10 l of 20 mM 2-[N-Morpholino]ethanesulfonic acid (MES), pH 7.0, 1 mM CaCl₂, 1% Triton X-100. After 20 minutes on ice, the sample was diluted 10 fold with 20 mM MES, 1mM CaCl₂ to bring the final concentration of Triton X-100 to 0.1% (furin cleavage buffer). The lysate was pelleted (16,000 g, 10 minutes, 4°C), and the supernatant collected and incubated in the presence or absence of 2 units of human furin (New England Biolabs, Beverly, MA) for 30 minutes at 37°C. The samples were analyzed by Western blotting and probed with rabbit anti-gp40 and anti-gp15 sera.

Cleavage of recEcgp40/15 and recTgpp40/15 by *T. gondii* lysate

ΔHXGPRT tachyzoites and excysted *C. parvum* oocysts were lysed in furin cleavage buffer (as described above) at a concentration of 10⁹ tachyzoites or oocysts/ml. The *E. coli* recombinant gp40/15 fusion protein (recEcgp40/15) was expressed as previously described [11] and purified by metal-affinity chromatography. recEcgp40/15 is a thioredoxin fusion protein with N- and C-terminal His tags for purification and an N-terminal S-tag for detection. 1g of recEcgp40/15 was incubated (overnight at 37°C) with either 2 units of furin, 30 l of *C. parvum* oocyst lysate, 30 l of ΔHXGPRT tachyzoite lysate, or the tachyzoite lysate plus 0.1mM decanoyl-RVKR-chloromethylketone (Bachem Biosciences, King of Prussia, PA), a specific furin inhibitor. Samples were analyzed by western blotting and probed with horseradish peroxidase-conjugated S-protein (Novagen, Madison, WI) to detect the S-tag in recEcgp40/15.

Tgpp40/15 expressing tachyzoites were also lysed in furin cleavage buffer as described and incubated overnight at 37°C in the presence or absence of 0.1mM decanoyl-RVKR-chloromethylketone. Western blots of the samples were probed with rabbit anti-gp40 serum to detect proteolytic cleavage of recTgpp40/15.

Results

T. gondii stable transfectants express *C. parvum* gp40/15 constructs

The four uncloned parasite lines transfected with pHLEMgp40/15, pHLEMgp40, pHLEMgp40HA, and pHLEMgp15 were 15, 10, 20 and 50% positive, respectively, for *C. parvum* gene expression after growth in selection medium. Cloning of the parasite lines by limiting dilution yielded 2–3 positive clones from each population that appeared identical in localization and expression as evaluated by IFA and western blot, respectively. Thus one clone of each population was selected for further evaluation. Only one positive clone was isolated from the population expressing recTggp40/15. Figure 1 shows localization and protein expression of the gp40/15 constructs in the stably transfected parasite clones. Localization was similar to that in transiently transfected parasites in that the proteins containing the GPI anchor signal (Tggp40/15 and Tggp15) localized to the tachyzoite membrane (Figure 1A), whereas the recTggp40 protein localized to an unidentified interior compartment (Figure 1A, Tggp40), that does not co-localize with dense granules, rhoptries or micronemes [25]. Addition of an HA tag to gp40 did not affect localization or protein expression (not shown). Both recTggp40/15 and recTggp40 glycoproteins displayed the carbohydrate epitope identified by mAb 4E9 (Figure 1A).

When the *T. gondii*-expressed recombinant proteins were examined by western blot analysis with rabbit anti-gp40 and gp15 sera, a single major band of the expected size and reactivity was expressed by the clonal populations (Figure 1B). Several minor bands in the Tggp40/15 lysate were recognized by anti-gp15 serum. The two lower molecular weight bands (Figure 1B, anti-gp15, lane 2, arrowheads) could represent gp15 processed from the precursor, but there was no evidence of the gp40 protein when Tggp40/15 lysates were probed with anti-gp40 serum (Figure 1B, lanes 2). The ~30kDa band (Figure 1B, arrows) in the Tggp40/15 lysate recognized by anti-gp40 and anti gp15 sera may be either a proteolytic degradation product of recTggp40/15, or a partially or non-glycosylated form of the protein. Anti-gp15 serum also consistently cross-reacts with a *T. gondii* protein of about 30kDa (Figure 1B lanes 3 and 4).

Purification of *T. gondii* expressed recombinant proteins

Since recombinant proteins purified away from *T. gondii* proteins were needed for further analysis we evaluated 2 different purification methods. The first purification method evaluated was anti-HA affinity chromatography of Tggp40-HA parasite lysates. The HA-tagged glycoprotein was specifically eluted from the αHA affinity column with an excess of HA peptide, and purification evaluated by HPA lectin blotting and silver staining of total protein (Figure 2). This method yielded a highly purified preparation of recTggp40-HA (Figure 2, lanes 3 and 4) as determined by silver staining. Because glycoproteins stain poorly with silver stain, a prolonged development time is required to visualize the glycoprotein band. Thus, the minor contaminant at 90kDa appears more prominent than it actually is. Infected cell lysates from five T150 flasks yielded approximately 75 g of recTggp40-HA.

Since native *C. parvum* gp40 can be purified by HPA lectin affinity chromatography [19], we also evaluated this purification strategy as a method for isolating significant quantities of untagged recTggp40/15. Large-scale cultures of HFF cells infected with Tggp40/15 parasites were lysed and the recombinant glycoprotein isolated by HPA lectin affinity chromatography (Figure 3). Evaluation of HPA purification by western blotting with mAb 4E9 demonstrated that all recTggp40/15 bound to the HPA column, and most of the protein was recovered by elution with 0.25M GalNAc (Figure 3, panel 4E9, lanes 1–3). Lectin blotting with HPA indicated that recTggp40/15 (Figure 3A, HPA panel, lanes 1–3) was free of contamination with other HPA reactive proteins (Figure 3, right panel, lane 1) and evaluation of total protein with

Sypro Ruby red stain demonstrated that recTgpp40/15 could be purified by this method. 25–30 g of recTgpp40/15 was purified from infected cell lysates collected from five T150 flasks.

Tgpp40-HA glycoprotein exhibits similar glycosylation patterns to native gp40

To determine if recombinant *C. parvum* glycoproteins produced in *T. gondii* exhibited similar glycosylation patterns to the native antigens, bands representing affinity purified recTgpp40-HA and gp40 from shed proteins were excised from SDS-PAGE gels and analyzed by liquid chromatography-electrospray mass spectrometry (LC-ESMS) and LC-ESMS/MS for the presence of sugars (Figure 4). In-gel-digestion of recTgpp40-HA and gp40 with trypsin resulted in a large number of peptides, resolved by LC-ESMS. Identification of the peptides from mass data using the MASCOT program confirmed their gp40 origin. Consequently, all major peaks in the mass spectrum were subjected to MS/MS. Manual search of the MS/MS spectra resulted in identification of double-, triple- and quadruple-charged glycosylated peptides. These glycopeptides were distinguished by the presence of sugar oxonium ions in the MS/MS spectra. Figure 4 shows fragmentation spectra of 1101.8 m/z and 1101.96 m/z parent ions corresponding to recTgpp40-HA and gp40, respectively. The identity of the glycopeptide could not be determined due to lack of peptide fragment ions. Oxonium ions were observed at m/z= 204.12 (HexNAc) and 366.19 (HexNAc-Hex) in recTgpp40-HA and gp40. In addition, ions arising from loss of H₂O (HexNAc-H₂O at m/z=186.10 and HexNAc-2H₂O at m/z= 168.07) were commonly seen on recTgpp40-HA and gp40 spectra (Figure 4). The relative intensities of the fragment ions are not quantitative in this experiment, but are indicative of glycosylation pattern. Because both proteins are recognized by HPA lectin (not shown), these peaks probably represent GalNAc and Galβ1–3GalNAc, Core 1 type O-glycans which are basic components of mucins. This data further corroborates the similarity in glycosylation patterns between the two glycoproteins.

Furin cleaves the recTgpp40/15 precursor

Previous work in our laboratory had determined that recEcgp40/15 is cleaved into gp40 and gp15 by recombinant human furin and by a furin-like enzyme activity present in lysates of *C. parvum* [21]. To determine if the gp40/15 precursor produced in *T. gondii* could be similarly cleaved, lysates of Tgpp40/15-infected HFF cells were incubated with furin and examined by Western blot (Figure 5). Furin cleaved recTgpp40/15 into the gp40 and gp15 products (Figure 5, lanes 3). Interestingly, the gp40 and gp15 products from furin-cleaved recTgpp40/15 were closer in size to the native proteins (Figure 5, lanes 1) than the recTgpp40 and recTgpp15 that were expressed individually (Figure 5, lanes 4a and 4b). These data suggest that appropriate post-translational modification may require expression of the full-length precursor. Anti-gp15 serum recognizes two low molecular weight bands in the Tgpp40/15 lysate cleaved with furin (Figure 5, panel B, lane 3), the larger of the two migrating identically to the native antigen. The smaller band is likely a degradation product, as longer incubations resulted in the disappearance of the gp15 bands (not shown).

***T. gondii* lysates contain furin-like protease activity that cleaves the gp40/15 precursor**

Anti-gp15 serum detects two low molecular weight bands on Western blots of Tgpp40/15 lysates (Figure 1B, anti-gp15 panel, lane 2 and Figure 5, panel B, lane 2) that are the same size as the bands appearing after furin-cleavage of recTgpp40/15 (Figure 5, panel B, lane 3). These observations suggested that protease activity capable of processing gp40/15 was present in *T. gondii*, but might require longer incubations under conditions favorable for furin activity. To determine if *T. gondii* expressed a furin-like enzyme capable of cleaving gp40/15, recEcgp40/15 was incubated overnight at 37°C with tachyzoites that had been lysed in furin cleavage buffer. Cleavage of the precursor was evaluated by western blotting with S-protein, which detects the N-terminal S-tag on the fusion protein. Overnight incubation of the precursor

with recombinant human furin results in complete cleavage of recEcgp40/15 (Figure 6A, lane 2). Protease activity present in both *C. parvum* (Figure 6A, lane 3) and *T. gondii* lysates (Figure 6A, lanes 4 and 5) also cleaves the recEcgp40/15, albeit incompletely. Cleavage of recEcgp40/15 by *T. gondii* lysate was blocked by the specific furin inhibitor, decanoyl-RVKR-chloromethylketone (Figure 6A, lane 6), suggesting that the protease activity in the *T. gondii* lysate was also furin-like. To corroborate these observations, a lysate of Tgpp40/15 tachyzoites was incubated overnight in the presence and absence of decanoyl-RVKR-chloromethylketone, and cleavage of the recTgpp40/15 evaluated by western blotting with rabbit anti-gp40 serum. In the absence of the furin inhibitor, anti-gp40 sera identified both the recTgpp40/15 precursor and a lower molecular weight band of the expected size for recTgpp40 (Figure 6B, lane 1). However, the recTgpp40 band was absent when the incubation was conducted in the presence of the furin inhibitor (Figure 6B, lane 2). These results suggest that a protease capable of processing gp40/15 is expressed by *T. gondii*, but the recombinant protein and the enzyme are in separate compartments in the intact parasite.

Because host cells can also be a source of furin-like activity, the tachyzoites were prepared for experiments evaluating furin-like activity in a manner that would minimize contamination from host cells. Tachyzoites were allowed to egress from HFF cells, and the free tachyzoites collected by slow speed centrifugation (400xg, 15 minutes). To evaluate the contribution of host cell protease activity, we mechanically disrupted uninfected HFF cells, and then centrifuged at 400xg. The pellet was solubilized in furin cleavage buffer as described, and incubated overnight at 37°C with 1 g of recE.coligp40/15. Lysate from uninfected HFF cells cleaved the recombinant protein (not shown), suggesting that furin contamination from host cells could account for the gp40/15 cleavage by tachyzoite lysate. Therefore, the source of the furin-like activity in the *T. gondii* lysates remains ambiguous.

The N-terminus of *T. gondii* expressed recombinant gp40 and gp40/15 is 11 amino acids upstream of the N-terminus of native gp40

The predicted cleavage site for the Cp40/15 signal peptide, as determined by the SignalP 3.0 program [30], is between amino acids 19 and 20 (VFS¹⁹⁻²⁰AP). However, the experimentally determined N-terminus of the mature protein is 11 base pairs downstream of this site between amino acids 30 and 31 (TLK³⁰⁻³¹DVP) [11] suggesting that the *C. parvum* signal peptidase either recognizes a longer signal sequence on gp40/15 or, that subsequent to signal sequence cleavage, further processing of the N-terminus to produce the mature antigen occurs. To determine if the *T. gondii*-expressed recombinants had the same N-terminus as native gp40, the N-terminus of affinity purified and gel isolated recTgpp40-HA was determined by sequencing. The protein sequence of recTgpp40-HA began at amino acid 20, at the predicted signal sequence cleavage site. Therefore, in addition to the HA tag, recTgpp40-HA bears additional N-terminal residues not present in mature native gp40.

To determine if the N-terminus differed when the full-length precursor was expressed, N-terminal sequence was obtained on HPA-purified recTgpp40/15. The N-terminus of recTgpp40/15 began at the predicted signal sequence (²⁰APA²²), not at the native *C. parvum* N-terminus (³¹DVP³³) as did recTgpp40-HA. These results suggested that the *T. gondii* signal peptidase either does not recognize the *C. parvum* signal sequence cleavage site, or does not express enzyme(s) which process the N-terminus or the enzyme/s are compartmentalized such that they do not have access to the recombinant *T. gondii* glycoproteins.

Discussion

T. gondii has often been proposed as a model for other apicomplexan parasites because it is easily propagated and it is amenable to genetic manipulation [31]. In particular, *T. gondii* is very attractive as a surrogate system to study aspects of *Cryptosporidium* biology as this

parasite, of all the medically important apicomplexans, presents the greatest challenges to laboratory experimentation. However, despite the theoretical advantages, actual examples of successful exploitation of this model system are few. Complementation of *T. gondii* Δ HXGPRT mutants with a *Cryptosporidium* library was used to identify the *Cryptosporidium* IMPDH gene [32]. The function of *Cryptosporidium* mitochondrial targeting sequences was confirmed by expression in *T. gondii* of fusion proteins bearing the putative targeting sequences [33]. When the cryptosporidial antigens, gp40/15 [25] and P23 [34], were expressed in *T. gondii*, the glycoproteins exhibited appropriate localization and glycosylation [25] and elicited anti-cryptosporidial antibodies in mice [34]. Additionally, *T. gondii* expressing the *Plasmodium yoelii* and *P. knowlseyi* CSP antigens has been used to elicit anti-CSP immune responses [35,36]. *T. gondii* has also been used extensively to validate cGMP-dependent protein kinase as a coccidial drug target [37,38].

Our studies suggest that *T. gondii* could be routinely used to express and study *Cryptosporidium* glycoprotein antigens. The generation of transfectants stably expressing the gp40/15 constructs enabled purification of sufficient amounts of recombinant protein to begin biochemical analysis. In addition to the transfected lines described here, we have also stably expressed CpMuc4 and CpMuc5, two other cryptosporidial mucin antigens belonging to a group of seven small mucin antigens expressed from a single locus on chromosome 2 (O'Connor and Ward, unpublished) as well as CpGalNAcT1, a cryptosporidial polypeptide N-acetylgalactosaminyl transferase (Bhat and Ward, unpublished). This system should prove particularly useful for the study of antigens present in low abundance as well as *C. hominis* antigens such as gp40/15 and gp900 that are often significantly divergent from their *C. parvum* orthologs [13,39,40]. Most *C. hominis* isolates cannot be propagated in calves [41], thus these parasites are not available for experimentation.

Our studies also highlight the obstacles that need to be overcome to generate recombinant gp40/15 that would successfully mimic native antigen. Processing of the gp40/15 precursor in *T. gondii* is not the same as in *Cryptosporidium*. Pulse-chase studies of gp40/15 processing in *C. parvum* have shown that proteolytic cleavage into gp40 and gp15 occurs almost coincident with protein synthesis [21], whereas processing in the Tggp40/15 transfectants is highly inefficient. This inefficiency may be due to sequestration of the recombinant antigen and protease to different compartments. Alternatively, *T. gondii* may not express a protease capable of cleaving gp40/15, and the observed cleavage may be the result of host cell furin activity. Studies in our laboratory have identified a furin-like subtilase activity in *Cryptosporidium* lysates that cleaves gp40/15 [21], but the subtilase responsible has not yet been identified. There is at least one subtilase gene in the *C. parvum* genome [15]. The catalytic domain of this gene, designated CpSUB1, is 45% homologous to the *Toxoplasma* subtilases, TgSUB1 and TgSUB2 [42,43]. TgSUB1, which localizes to the micronemes, and TgSUB2, which is found in the rhoptries, are thought to be involved in processing of the apical complex proteins involved in invasion. Interestingly, in gp40/15 the sequence SLSE, that occurs directly adjacent to and downstream of the RSRR furin site, conforms to the S ϕ XE motif thought to be the recognition site for TgSub2 cleavage [42]. It may be possible to target recTggp40/15 to the micronemes [44] or rhoptries [45,46] to facilitate efficient processing of recTggp40/15 and determine whether gp40/15 is a substrate for TgSUB1 and TgSUB2.

Interestingly, N-terminal sequencing of the recTggp40/15 antigen revealed that the N-terminus begins 11 amino acids upstream of the N-terminus of the native antigen at the site predicted to be the signal sequence cleavage site. This observation raises the issue of how *Cryptosporidium* processes the N-terminus of gp40. *Cryptosporidium* may recognize the whole 30 amino acids as a signal sequence or the signal sequence may be cleaved as predicted and the N-terminus further processed by other proteases possibly as part of an activation step. This processing could occur within the secretory compartments (propeptide processing) or on the

surface (primary processing) as has been described for other apicomplexan antigens [47]. For example, the N-terminus of *TgMIC2* is cleaved off by the protease MPP2 as the *TgMIC2*/M2AP complex localizes to the tachyzoite surface [48]. Comparisons of rec*Tggp40/15* expressed with and without the 11 amino acid terminus may yield insight into how and when N-terminal processing of gp40/15 occurs.

Our previous studies indicated that glycosylation of gp40/15 in *C. parvum* and *T. gondii* was highly similar; the *T. gondii* recombinant exhibited the 4E9 epitope (specific to gp40 and gp900), reacted with the α GalNAc specific lectin HPA, and displayed a change in M_r upon digestion with α -N-acetyl galactosaminidase, an enzyme specific for terminal α GalNAc residues [25]. These studies confirm and extend those observations. Mass spectrometric analysis of native and rec*Tggp40*-HA demonstrated the presence of oxonium ions at m/z =204.12 (HexNAc) and 366.19 (HexNAc-Hex) in both glycoproteins, that most likely represent α GalNAc and Gal β 1-3GalNAc in light of the reactivity with HPA. Further analysis is needed to compare the actual structures of the oligosaccharides on the native and recombinant antigens. This analysis would best be done with rec*Tggp40* isolated from furin-cleaved precursor, which appeared to be closer in size to the native antigen than the gp40 expressed alone. The difference in molecular weight is probably due to glycosylation differences, as the N-terminus was the same in both recombinants, and other studies have shown that recombinant human furin consistently cleaves gp40/15 at RSRR↓.

These studies illustrate the practicality of the *T. gondii* expression system for production of *Cryptosporidium* antigens. Both HPA and HA affinity purification methods yielded significant quantities of highly purified recombinant glycoproteins. Because antigen glycosylation can impact protein function by affecting protein folding, interactions with other proteins, recognition of host cell receptors, and additionally may impact immune system recognition and response, the ability to produce purified recombinant glycoproteins similar to the native antigens opens several areas of investigation in *Cryptosporidium* biology. These reagents are essential tools for identification of host cell receptors, particularly for gp40/15. gp40/15 exhibits extensive polymorphism among *C. hominis* isolates [13,23] that could impact recognition of different host cell receptors. Indeed, many putative mucin antigens present in the *C. hominis* genome exhibit significant diversity from their *C. parvum* orthologs [16], and the functional significance of this diversity remains unexplored. *Cryptosporidium* glycoprotein antigens produced in *T. gondii* could provide an essential tool for investigation of these questions, furthering the goal of developing effective therapeutics and vaccines.

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Abbreviations

GPI	glycophosphatidyl inositol
MWCO	molecular weight cut-off
HFF	human foreskin fibroblast
HXGPRT	hypoxanthine-xanthine-guanine phosphoribosyl transferase
mAb	monoclonal antibody
HPA	<i>Helix pomatia</i> lectin
HA	hemagglutinin
IFA	immunofluorescence assay

Figure 1. Stably transfected *T. gondii* clones expressing gp40/15, gp40 and gp15

A: IFA's of stably transfected *T. gondii* clones. The *Cryptosporidium* proteins (green) are co-localized with SAG-1 (red). Scale bars = 100m. **B:** Lysates of infected HFF cells were analyzed by western blotting with rabbit antisera to gp40 and gp15. Cells were infected with *Tg*gp40/15 (lanes 2), *Tg*gp40 (lanes 3) and *Tg*gp15 (lanes 4). *C. parvum* oocyst lysates are included for comparison (lanes 1). Molecular weights are shown on left.

Figure 2. HA-affinity purification of recTgpp40-HA

Tgpp40-HA parasite lysate was reacted with HA-agarose. recTgpp40-HA was specifically eluted with 1mg/ml of HA peptide. Purification was assessed by western blotting with HPA lectin (left panel) and silver stain (right panel) Lane 1: Pre-column lysate; lane 2: column flow through; lane 3: eluate; lane 4: silver stain of eluate concentrated by ultrafiltration (30Kda MWCO). Molecular weight markers shown on the left.

Figure 3. HPA affinity purification of *recTgpp40/15*

Parasites expressing *recTgpp40/15* were lysed and reacted with HPA-agarose. Glycoproteins were eluted with 0.25M N-acetylgalactosamine. **Panel A:** Purification was evaluated by western blotting with mAb 4E9 and HPA lectin. Lanes 1: pre-column lysate; lanes 2 column flow through; lanes 3: eluate. **Panel B:** Contamination of purified proteins was evaluated by Sypro Ruby red staining of SDS-PAGE gels: lane 1: purified *recTgpp40/15*, Arrow indicates the *recTgpp40/15* glycoprotein band.

Figure 4. Tryptic fragments from *recTgpp40*-HA and native gp40 contain the same sugars
Purified *recTgpp40*-HA and gp40 were gel-isolated and subjected to tryptic digest. The peptides were separated by C18 reversed-phase HPLC and analyzed on a mass spectrometer. The ions at 1101.8 m/z in *recTgpp40* and 1101.96 m/z in native gp40 were selected for MS/MS fragmentation based on charge and intensity, and then subjected to low energy collision-induced decay for 8s to produce the characteristic fragment ions shown in the figure. Because the run was automated, conditions were not optimized for individual ions. Fragment ions m/z=168.07 (HexNAc-2H₂O), m/z=186.1 (HexNAc-H₂O), m/z=204.12 (HexNAc), and m/z=366.19 (HexNAc-Hex) are present in both samples. The relative intensities of the fragment ions are not quantitative in this experiment. Because both peptides have the same intact mass, the mass of the glycan moiety is assumed to be the same.

Figure 5. Furin cleaves Tgrgp40/15 into gp40 and gp15

Lysates of transfected parasites were incubated with or without furin and analyzed by western blotting with rabbit antisera to gp40 (panel A) and gp15 (panel B). Lanes 1: *C. parvum* oocyst lysates; lanes 2: Tggp40/15 lysate; lanes 3: Tggp40/15 lysate with furin; lane 4a: Tggp40 lysate; lane 4b: Tggp15 lysate. Molecular weight markers are shown on left.

Figure 6. Lysates of *Toxoplasma* tachyzoites contain the furin-like protease activity that cleaves the gp40/15 precursor

Panel A: Western blots of recEcgp40/15 fusion protein (lane 1) incubated overnight with recombinant human furin (lane 2), *Cryptosporidium* lysate (lane 3), *Toxoplasma* lysate (lanes 4 and 5), and *Toxoplasma* lysate plus the furin inhibitor decanoyl-RVKR- chloromethylketone (lane 6). Proteins were detected by means of the N-terminal S-tag. **Panel B:** Western blots of lysates of Tggp40/15 tachyzoites incubated in the absence (1) and presence (2) of decanoyl-RVKR- chloromethylketone. Blots were probed with rabbit α gp40-serum. MW standards are indicated on the left.

Table 1

Cloning primers

Name	Sequence [*]
Signal Nsi F	5'-GGA TGC ATA GAT TGT CGC TCA TTA TCG TA-3'
gp40 Pac R	5'-GGT TAA TTA ACG TGA TGA AGC CTG ACC CGC AGA TCC-3'
gp40HA Pac R ^{**}	5'-GGT TAA TTA AGC GTA ATC TGG AAC ATC GTA TGG GTA CTC TGA GAG TGA TCT TCT TGA TCT TG-3'
gp15 Pac R	5'-GGT TAA TTA AAG CAC GAA TAA GGC TGC AAA G-3'
HindIII R	5'-GGC TAC CAG AAG CTT CAG TAC-3'
HindIII F	5'-GGT ACT GAA GCT TCT GGT AGC-3'
AluI R	5'-GGT TGC AGC AGC TTC ACT GGT-3'
AluI F	5'-GTG AAG CTG CTG CAA CCG TCG-3'

* restriction sites in bold;

** coding sequence for HA tag is boxed