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Biochemical and functional characterization of CpMuc4, a *Cryptosporidium* surface antigen that binds to host epithelial cells

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Abstract

Cryptosporidium spp. are intracellular apicomplexan parasites that cause outbreaks of waterborne diarrheal disease worldwide. Previous studies had identified a *C. parvum* sporozoite antigen, CpMuc4, that appeared to be involved in attachment and invasion of the parasite into intestinal epithelial cells. CpMuc4 is predicted to be O- and N-glycosylated and the antigen exhibits an apparent molecular weight 10kDa larger than the antigen expressed in *E. coli*, indicative of post-translational modifications. However, lectin blotting and enzymatic and chemical deglycosylation did not identify any glycans on the native antigen. Expression of CpMuc4 in *T. gondii* produced a recombinant protein of a similar molecular weight to the native antigen. Both purified native CpMuc4 and *T. gondii* recombinant CpMuc4, but not CpMuc4 expressed in *E. coli*, bind to fixed Caco-2A cells in a dose dependent and saturable manner, suggesting that this antigen bears epitopes that bind to a host cell receptor, and that the *T. gondii* recombinant CpMuc4 functionally mimics the native antigen. Binding of native CpMuc4 to Caco2A cells could not be inhibited with excess CpMuc4 peptide, or an excess of *E. coli* recombinant CpMuc4. These data suggest that CpMuc4 interacts directly with a host cell receptor and that post-translational modifications are necessary for the antigen to bind to the host cell receptor. *T. gondii* recombinant CpMuc4 may mimic the native antigen well enough to serve as a useful tool for identifying the host cell receptor and determining the role of native CpMuc4 in host cell invasion.

Keywords

Cryptosporidium; *Toxoplasma*; glycoproteins; sporozoite; attachment; adhesins

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1. Introduction

Cryptosporidium spp. are ubiquitous waterborne pathogens that cause diarrheal disease worldwide. These parasites have been frequently identified as the etiological agents of diarrheal disease outbreaks associated with contaminated drinking and recreational waters in developed countries [1]. In developing countries where these parasites are endemic, they are the most common cause of parasitic diarrhea in children under the age of five [2]. Cryptosporidiosis was recently found to be second only to rotavirus as the primary cause of diarrhea in infants and one of the two enteric pathogens associated with death in toddlers in Africa and Asia [3]. Even when the infection is asymptomatic, the morbidity associated with cryptosporidiosis is significant, particularly when exacerbated by malnutrition or HIV infection [4]. However, identification of virulence factors that could be exploited in vaccine design has lagged far behind that of other apicomplexan parasites, likely due to the experimental intractability of these parasites.

Unlike other apicomplexans, glycoproteins appear to play an essential role in the ability of *Cryptosporidium* spp. sporozoites to attach and invade intestinal epithelial cells [5-9]. Lectins and monoclonal antibodies reactive with these glycoprotein antigens inhibit infection in vitro [7,10] and in vivo [11-17], suggesting that these antigens are potential vaccine candidates. In a previous study, we mined the *C. parvum* and *C. hominis* genome databases [18-20] for secreted glycoproteins and identified a locus on chromosome 2 encoding seven putative mucin-like glycoproteins [21]. One of these, that we termed Muc4, exhibited extensive polymorphisms between the *C. parvum* and *C. hominis* alleles [21]. Subsequently, we identified other Muc4 allelic forms in clinical samples [21]. *C. parvum* (Cp)Muc4 was found to localize to the apical surface of sporozoites. The apparent molecular weight of native (n)CpMuc4 of 30 kDa, 10 kDa larger than the predicted molecular weight, as well as the presence of several predicted glycosylation sites, indicated extensive post-translational modification of the native antigen [21]. The importance of this antigen to host – parasite interactions was underscored by the observation that a polyclonal antibody specific for a single CpMuc4 peptide could inhibit *C. parvum* infection of Caco2A cells [21]. Furthermore, nCpMuc4 was shown to bind to intestinal epithelial cells in a dose-dependent and saturable manner [21]. These data suggested that nCpMuc4 is a sporozoite surface glycoprotein that plays an essential role in parasite attachment and invasion. However, binding of nCpMuc4 to host epithelial cells had been detected using oocyst lysates, so it could not be ascertained if the nCpMuc4 antigen directly interacted with a host cell receptor, or if it was part of a complex of proteins binding to the host cell.

Since *C. parvum* cannot be propagated in vitro, or genetically manipulated, further exploration of the role of this antigen in host-pathogen interactions presented significant challenges. nCpMuc4 can be isolated in limited amounts from *C. parvum* oocysts, but identification of the specific epitopes involved in host cell interactions, and the impact of post-translational modifications, such as glycosylation, on antigen function would require generation of a recombinant antigen that would mimic the function of the native antigen. We had previously demonstrated that *C. parvum* glycoproteins expressed in *Toxoplasma gondii* were appropriately post-translationally modified, and might be expected to retain the function of the native antigen [22,23]. In the studies described here, we affinity purify

nCpMuc4 and demonstrate that the isolated antigen binds to intestinal epithelial cells suggesting that nCpMuc4 does interact directly with a host cell receptor. Furthermore, we show that recombinant CpMuc4, produced in *T. gondii*, mimics the host cell binding properties of the native antigen, whereas CpMuc4 produced in *E. coli* does not bind to host cells. These results suggest that post-translational modifications are important for antigen function, but, while nCpMuc4 is predicted to be O- and N-glycosylated, these studies failed to identify any glycans on nCpMuc4.

2. Methods and Materials

2.1 Parasites

C. parvum Iowa isolate oocysts were purchased from Bunch Grass Farm (Deary, ID). For preparation of soluble oocyst lysate, oocysts were suspended at 10^9 /ml in phosphate buffered saline (PBS) plus protease inhibitors (Protease Inhibitor Cocktail Set III, Calbiochem, La Jolla, CA) and sonicated on ice for 20 to 30 minutes using 2, 15 second pulses per minute, until greater than 90% of the oocysts were lysed. Oocyst lysate was centrifuged at $16000\times g$ for 30 minutes to remove insoluble material. *T. gondii* RH strain parasites lacking the hypoxanthine-xanthine-guanine phosphoribosyl transferase (HXGPRT) gene were obtained from Dr. David Roos, University of Pennsylvania [24] and maintained in human foreskin fibroblast (HFF) cells.

2.2 Antibodies

Generation of rabbit anti-CpMuc4 antibody, rabbit anti-CpMuc4 peptide antibody [21], and rabbit anti-gp40 antibody [25] has been described. Briefly, rabbit anti-CpMuc4 antibody was generated by immunizing rabbits with the full length CpMuc4 protein expressed in *E. coli* as a thioredoxin fusion protein (Harlan Bioproducts for Science, Indianapolis, IN). Anti-CpMuc4 peptide antibody was raised against the CpMuc4 peptide 102 PNPFAGVSLSSPRPR 116 coupled to keyhole limpet hemocyanin. The anti-CpMuc4-peptide IgG was affinity purified from the sera on a peptide Sepharose column (Dragonfly Sciences, Wellesley, MA) [21]. Anti-SAG1 mAb DE52 was obtained from Dr Marc-Jan Gubbels, Boston College.

2.3 Immunoassays

Parasite lysates and purified proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% acrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes. Western blotting to detect native and recombinant CpMuc4 proteins was conducted as previously described using rabbit anti-CpMuc4 antibody [8]. For indirect immunofluorescence assays (IFAs), *T. gondii* intracellular stages were fixed with 4% paraformaldehyde, permeabilized with 0.25% Triton X-100 and probed with primary antibodies to SAG-1 and CpMuc4 [26]. Primary antibodies were detected with Alexa Fluor conjugated secondary antibodies as appropriate (Life Technologies, Grand Island, NY). 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 performed using Volocity software (Perkin Elmer, Waltham, MA).

2.4 Affinity purification of nCpMuc4

Soluble oocyst lysate was combined with rabbit anti-CpMuc4 peptide antibody (at a final concentration of 75 µg/ml) and incubated for two hours at room temperature. The lysate-antibody mixture was then added to Protein G magnetic beads (Dynabeads, Life Technologies) and incubated for 45 minutes at room temperature. The lysate was collected off the beads ("flow-through") and the beads washed extensively with phosphate buffered saline at pH 7.4 (PBS). The nCpMuc4 antigen was eluted with 20 mg/ml of the CpMuc4 peptide (¹⁰²PNPFAGVSLSSPRPR¹¹⁶) diluted in PBS, pH7.4 at 37°C for 20 minutes. Complete antigen recovery was achieved with three elutions. Elutions were pooled and the CpMuc4 peptide removed by ultrafiltration in Microcon units (10,000 molecular weight cut-off; EMD Millipore, Billerica, MA).

2.5 Liquid chromatography tandem mass spectrometry (LC/MS/MS)

Purified nCpMuc4 was resolved on SDS-PAGE gels, stained with Coomassie Brilliant Blue R250 and excised. The band containing the antigen was subjected to in-gel digestion with trypsin or a combination of trypsin, chymotrypsin and pepsin. The LC/MS/MS data was collected with a Thermo LTQ ion trap mass spectrometer (Tufts Proteomics Core) and a SEQUEST [27] search of the LC/MS/MS data was done against the NCBI non-redundant protein database (<ftp://ftp.ncbi.nih.gov/blast/db/FASTA/nr>) and the CpMuc4 sequence (CryptoDB gene ID# cgd2_420) .

2.6 Lectin blotting

Oocyst lysate and purified nCpMuc4 were resolved by SDS-PAGE, blotted to PVDF membranes and probed with the biotinylated lectins listed in Table 1 (EY Laboratories, Inc; San Mateo, CA). Lectin binding was detected with alkaline phosphatase (AP) conjugated streptavidin (Vectastain ABC-AP kit, Vector Laboratories) and 5-bromo-4-chloro-3'-indolylphosphate/ nitro-blue tetrazolium chloride (BCIP/NBT) substrate (Vector Laboratories). To determine the specificity of UEA-1 and Jacalin reactivity with nCpMuc4 western blots, blots were probed with the lectins combined with an excess of the cognate sugar (200mM fucose and melibiose, respectively). Rabbit anti-CpMuc4 antibody, detected with AP-conjugated goat anti-rabbit IgG, was run in parallel to confirm the presence of sufficient nCpMuc4 on the blots.

2.7 Enzymatic deglycosylation

Purified nCpMuc4 was digested with peptide -N-glycosidase F (PNGase-F), α -fucosidase , α N-acetyl galactosaminidase, and a protein deglycosylation mixture consisting of PNGase-F, O-glycosidase, neuraminidase, β1-4 galactosidase, and β-N-acetylglucosaminidase (all enzymes and Protein Deglycosylation Mix from New England Biolabs (NEB), Ipswich, MA). nCpMuc4 incubated in the digestion buffers in the absence of the enzymes was run in parallel as a control. The activity of the enzymes in the Protein Deglycosylation Mix was

confirmed by digestion of fetuin, an N- and O-glycosylated protein included in the protein deglycosylation kit as a positive control (NEB). Digestions were performed overnight at 37°C following the manufacturer's directions. The effect of the glycosidases on the apparent molecular weight of nCpMuc4 was evaluated by western blot.

2.8 Chemical deglycosylation

Oocyst lysate or purified nCpMuc4 was dried down in a glass vial and digested with trifluoromethanesulfonic acid (TFMS) for 4 or 16 hours at 4°C following manufacturer's directions (Glyco Profile IV Chemical Deglycosylation kit, Sigma-Aldrich). Anisole was included in the reaction as a free radical scavenger to prevent protein degradation. At the end of the incubation, 60% pyridine was added until the pH of the reaction mixture reached 6.0. The sample was dialyzed overnight against PBS and deglycosylation of nCpMuc4 was evaluated by western blot. RNaseA was run in parallel as a positive control. When deglycosylation was performed on oocyst lysate, western blots of deglycosylated lysate were also probed with antibody to the known *C. parvum* glycoprotein, gp40 [25], to confirm deglycosylation of parasite glycoproteins had occurred.

2.9 Expression of CpMuc4 in *T. gondii*

The coding sequence for nCpMuc4, starting at amino acid 31, was fused to the signal sequence from *T. gondii* GRA1 and cloned into the *T. gondii* expression vector pHLEM under the control of the GRA1 promoter and upstream of the GRA2 3'UTR. This vector also contains the *T. gondii* hypoxanthine-xanthine-guanine phosphoribosyl transferase (HXGPRT) gene under the control of the *T. gondii* dihydrofolate reductase gene regulatory elements for the generation of stable transfectants [24]. pHLEM was obtained from Dr. David Roos, University of Pennsylvania. Supplemental Table 1 lists the primers used for cloning. The forward primer incorporates an NsiI cloning site and the coding sequence for the GRA1 signal sequence (NsiI F-GRA1ss-CpMuc4). The reverse primer contains a PacI cloning site that provides the stop codon (Supplemental Table 1; PacICpMuc4-R). The PCR product was amplified from *C. parvum* oocyst gDNA, cut with NsiI (NEB) followed by PacI (NEB) and ligated into the NsiI-PacI cut vector. The ligation mixture was used to transform *E. coli* Top10 cells (Life Technologies). The transformants were selected on LB-ampicillin plates. The insert sequence was verified and the plasmid (designated pHLEM-GRA1CpMuc4) containing the correct sequence was purified using a Qiagen Megaprep Kit (Qiagen, Valencia, CA).

T. gondii tachyzoites were transfected with 50 µg of pHLEM-GRA1CpMuc4 as previously described [28] using a Biorad Gene Pulser II electroporator (Bio-Rad, Hercules, CA) at settings of 1.9 kV and 50 µF capacitance. After a 10-minute incubation at room temperature, the parasites were added to HFF monolayers in 25 mm² flasks and the transfectants selected and maintained in Dulbecco's minimum essential medium (DMEM) containing 10% fetal calf serum, 25 mM HEPES, 2X L-glutamine, 1X penicillin/streptomycin, 50 µg/ml xanthine and 25 µg/ml of mycophenolic acid (selection medium) [24].

To prepare a soluble lysate of *T. gondii* parasites expressing CpMuc4, free tachyzoites were collected from large scale cultures, pelleted and resuspended in PBS plus protease inhibitors.

Tachyzoites were sonicated for 5 minutes on ice as described above, and centrifuged at 16,000×g to remove insoluble material. Recombinant *T. gondii*-expressed CpMuc4 (rTgCpMuc4) was affinity-purified following the protocol developed for nCpMuc4.

2.10 Binding assays

Caco-2A cells were grown to confluence in 96-well plates, fixed with 1% glutaraldehyde, and blocked with 0.1 M glycine, followed by 0.1% BSA. To standardize the concentration of the nCpMuc4 and rTgCpMuc4, western blots were run of nCpMuc4, rTgCpMuc4, and known concentrations of CpMuc4 expressed in *E. coli* (rEcCpMuc4; produced as described in [29]). Concentrations of nCpMuc4 and rTgCpMuc4 were estimated by densitometry by comparison to the standard curve generated with rEcCpMuc4. Purified proteins, diluted to equal concentrations in Dulbecco's Modified Eagle Medium (DMEM) containing 25mM HEPES and 0.1% BSA, were added to the cells in a 50 µl volume, and incubated overnight at 4°C. The cells were washed three times with DMEM, and the bound proteins fixed to the cells with methanol. Binding of nCpMuc4 and rTgCpMuc4 to the host cells was detected with anti-CpMuc4 peptide antibody. The primary antibody was detected with biotinylated goat anti-rabbit IgG and alkaline phosphatase-labeled ABC reagent (Vector Laboratories) as described [8]. The binding of nCpMuc4 and rTgCpMuc4 to the cells fit a binding saturation curve calculated using the one site-specific binding equation in GraphPad Prism (La Jolla, CA). Bmax and Kd were calculated from this equation and a Scatchard plot generated.

To evaluate binding of the native antigen in the presence of rEcCpMuc4 or the CpMuc4 peptide, Caco2A cells were grown to confluence in 96 well plates, fixed with glutaraldehyde and blocked. Cells were incubated with nCpMuc4 (approximately 50 ng/well) combined with rEcCpMuc4 (1 µg/well), CpMuc4 peptide (10 µg/well) or buffer overnight at 4C. Unbound proteins were washed off, the cells were lysed in SDS-PAGE sample buffer and the binding of nCpMuc4 to the host cells was evaluated by western blotting.

2.11 Biotinylation of rTgCpMuc4

Purified rTgCpMuc4 was biotinylated using the SureLink Chromophoric biotin labeling kit (Kirkegaard & Perry Laboratories, Inc; Gaithersburg, MD) following the manufacturer's guidelines. Before biotinylation, contaminating BSA was removed by ultrafiltration through a 50,000 MWCO Microcon, followed by concentration of the rTgCpMuc4 in a 10,000 MWCO microcon. rTgCpMuc4 was quantified by densitometry as described for nCpMuc4. Biotinylation of rTgCpMuc4 was confirmed by western blotting and detection with Vectastain ABC-AP kit and BCIP/NBT substrate. Biotinylated rTgCpMuc4 was incubated with fixed Caco2A cells as described above, and binding to the cells detected with the ABC-AP kit and p-nitrophenyl phosphate (pNPP, Vector Laboratories).

3. Results

3.1 LC/MS/MS analysis of affinity purified nCpMuc4

nCpMuc4 was isolated from lysates of soluble oocyst proteins (Fig. 1B, arrow) using an affinity purification strategy involving capture of the antigen with the monospecific anti-CpMuc4 peptide antibody, and elution with an excess of the CpMuc4 peptide (Fig. 1A). The

prominent high molecular weight band that was a major contaminant in all the purifications was identified by N-terminal sequencing to be BSA (Fig. 1B, asterisk). Since N-terminal sequencing of nCpMuc4 was unsuccessful, the identity of the isolated protein was confirmed by LC/MS/MS of the tryptic peptides (Fig. 1C and Table 2). This approach confirmed that the isolated protein was nCpMuc4, but the peptides identified provided only 42% coverage. In order to increase coverage, an overnight in gel digestion with a mixture of pepsin, chymotrypsin and trypsin was performed, but with this approach very few peptides were identified with high confidence (Table 3; Sf scores above 0.8 [27]).

For the purposes of expressing CpMuc4 in *T. gondii*, it was desirable to identify the true N-terminus of the native protein. N-terminal sequencing of gp40, another *Cryptosporidium* surface glycoprotein, had demonstrated that the actual N-terminus was 11 amino acids downstream of the predicted signal sequence cleavage site, suggesting that the N-terminus of gp40 was proteolytically processed to produce the mature protein [8,22]. Proteolytic processing of the gp40 N-terminus did not occur when this antigen was expressed in *T. gondii* [22]. Although they were not identified with high confidence, three of the peptides identified in the second LC/MS/MS analysis (Table 3, italicized) started at amino acid 30 of the CpMuc4 sequence (Fig. 1C, star), 10 amino acids downstream of the predicted N-terminus [30]. When we expressed CpMuc4 in *T. gondii* we used amino acid 30 as the N-terminus of the recombinant protein.

3.2 Post-translational modifications of nCpMuc4

nCpMuc4 is predicted to be O-glycosylated (Fig. 1C, residues indicated in bold) and N-glycosylated (Fig. 1C, italicized residue), and has an apparent molecular weight of 30 kDa, approximately 10 kDa greater than its predicted molecular weight of 19.7kDa. rEcCpMuc4 migrates at the expected molecular weight of approximately 20 kDa (Fig. 2B, lane 4) suggesting that the polypeptide does not run anomalously as might be the case for a protein with a high proline content (11%). Several approaches were taken to characterize nCpMuc4 glycosylation. The reactivity of nCpMuc4 with a panel of lectins specific for O- and N-glycans (Table 1) was investigated. Lectins reacted with oocyst lysate as previously described [31] but none of the lectins tested reacted with purified nCpMuc4 (Supplemental Fig. 1A). Rabbit anti-CpMuc4, run in parallel as a positive control for the lectin blots, reacted strongly with nCpMuc4 confirming that there was sufficient antigen on the blots to be detected (Supplemental Fig1A, lane 1). Likewise, digestion with glycosidases specific for N- and O-linked glycans had no effect on the molecular weight of nCpMuc4 as determined by western blot and detection of the antigen was unaffected by glycosidase treatment (Supplemental Fig 1B). Since purified nCpMuc4 did not survive the desalting and drying required for chemical deglycosylation, oocyst lysate was digested with TFMS and the effect on nCpMuc4 evaluated by Western blot (Supplemental Fig. 2). TFMS treatment for 4 hours had no effect on the apparent molecular weight of nCpMuc4 (Supplemental Fig. 2, lanes 1 and 2), although gp40, another *C. parvum* glycoprotein, was partially deglycosylated as indicated by a shift in the molecular weight of the antigen (Supplemental Fig. 2, lanes 3 and 4). When the incubation was extended for 16 hours, neither nCpMuc4 or gp40 could be detected by western blot suggesting that the antigens had been degraded by the extended treatment (not shown).

3.3 Stable expression of CpMuc4 in *T. gondii*

CpMuc4 was cloned and expressed in *T. gondii* [22,23]. To achieve stable expression of CpMuc4 in *T. gondii*, the native CpMuc4 signal sequence was replaced with the signal sequence from *T. gondii* GRA1. After growth in selection medium, most of the tachyzoites were positive for rTgCpMuc4 expression (>90%), and this mixed population was used for antigen purification. rTgCpMuc4 localized to the cytoplasm of tachyzoites (Fig. 2A), and was purified from tachyzoite lysates using the same affinity purification strategy as for the native antigen. In contrast to rEcCpMuc4 (Fig. 2B, lane 4), rTgCpMuc4 (Fig. 2B, lane 1) exhibited nearly the same molecular weight as the native antigen (Fig. 2B, lanes 2 and 3), suggesting that appropriate post-translational modifications had occurred.

3.4 Binding of purified nCpMuc4 and rTgCpMuc4

To determine if nCpMuc4 contained epitopes that interacted directly with receptor(s) in intestinal epithelial cells, the ability of purified nCpMuc4 to bind to glutaraldehyde-fixed Caco2A cells was tested. Purified nCpMuc4 bound to the cells in a dose-dependent and saturable manner (Fig. 3). rTgCpMuc4 bound to Caco2A cells in a nearly identical pattern to that of the native antigen (Fig. 3), whereas rEcCpMuc4 bound at much lower levels (Fig. 3). The binding of nCpMuc4 and rTgMuc4 (but not binding of rEcCpMuc4) to Caco2A cells fit a saturation curve from which kinetic parameters were calculated (Fig 3) and a Scatchard plot generated (Supplemental Fig. 3).

To determine if either rEcCpMuc4 or the CpMuc4 peptide could block binding of nCpMuc4 to Caco2A cells, we tested the ability of nCpMuc4 to bind in the presence of excess rEcCpMuc4 or CpMuc4 peptide. Binding of nCpMuc4 was detected by Western blot (Fig. 4). Binding of rEcCpMuc4 to Caco2A cells could not block the binding of nCpMuc4, even when the recombinant antigen was added in great excess (Fig. 4, lanes 8 and 9), suggesting that the rEcCpMuc4 binding is very low affinity compared to the native antigen, or is non-specific. Because antibody specific for the CpMuc4 peptide inhibited *C. parvum* infection of Caco2A cells [21], we also tested the ability of the CpMuc4 peptide to inhibit binding of nCpMuc4, but an excess of the peptide likewise had no effect on nCpMuc4 binding to host cells (Fig. 4, lanes 6 and 7). Since rTgCpMuc4 and nCpMuc4 are of nearly identical molecular weight, this approach could not be used to determine if rTgCpMuc4 could block nCpMuc4 binding to host cells. We could not obtain sufficient nCpMuc4 antigen to directly label the antigen for detection. In order to distinguish between rTgCpMuc4 and nCpMuc4 in a competition experiment, rTgCpMuc4 was biotinylated. However, biotinylation of rTgCpMuc4 destroyed the antigen's ability to bind to Caco2A cells, precluding a direct comparison of the receptor specificity of nCpMuc4 and rTgCpMuc4.

4. Discussion

Our previous work demonstrated that nCpMuc4 plays a critical role in the attachment and invasion of *Cryptosporidium* sporozoites into host cells [21]. nCpMuc4 is found on the apical surface of sporozoites and antibodies reactive with surface epitopes of this antigen inhibit infection in vitro. Furthermore, nCpMuc4 is highly polymorphic between species and among clinical isolates [21], a characteristic associated with important virulence factors in *T.*

gondii [32,33]. In these studies we further demonstrate that nCpMuc4 interacts directly with host cells, although the receptor remains unknown. Invasion of *C. parvum* into host cells is perhaps more accurately described as engulfment [34,35]. After attachment, the parasite initiates host cytoskeleton reorganization by recruiting both phosphatidylinositol 3-kinase [36,37] and c-src [38] to the invasion site and through these pathways activating actin polymerization. Analysis of the CpMuc4 sequence and the *C. hominis* Muc4 polymorphic variants (accession #s FJ184998 to FJ185010) with the Eukaryotic Linear Motif (ELM) program [39] identified the presence of NPF and/or GPF motifs in all the sequences. These motifs interact with EH-domain containing proteins that are involved in endocytic processes [40-43]. This observation suggests that nCpMuc4 may play a role in the initiation of host cell responses that result in parasite engulfment, and encourages further investigation into the identity of the host cell receptor.

In the CryptoDB database [19], CpMuc4 is identified as a mucin-like glycoprotein on the basis of the high proline, threonine and serine content of the sequence and the presence of predicted O-glycosylation sites. Furthermore, the apparent molecular weight of nCpMuc4 on SDS-PAGE gels is 30 kDa, 10 kDa greater than that of the *E. coli* recombinant protein (Fig. 2B). This data argues for post-translational modification of this antigen by glycosylation, and bioinformatics analysis of the CpMuc4 sequence provides no other obvious explanation for the molecular weight discrepancy. Furthermore, while rEcCpMuc4 exhibits some binding to Caco2A cells (Fig. 3 and Fig. 4), binding of the nCpMuc4 antigen to host cells was not inhibited in the presence of excess rEcCpMuc4, suggesting that post translational modification of the antigen was necessary for recognition of the host cell receptor. Yet all the approaches applied in these studies to characterize glycotopes on this antigen failed. Lectins specific for N- and O-linked glycans did not recognize the antigen on western blots (Supplemental Fig. 1A), digestion with N- and O- glycosidases had no effect on the molecular weight of the antigen (Supplemental Fig. 1B) and chemical deglycosylation, performed under conditions that deglycosylated the glycoprotein gp40, had no effect on the CpMuc4 polypeptide (Supplemental Fig. 2). Monosaccharide and polysaccharide analysis also failed to detect any glycans (not shown), but this could have been attributed equally to insufficient antigen for analysis as to an absence of antigen glycosylation. Additionally, the peptides identified with high confidence by LC/MS/MS encompass 5 of the 8 O-glycosylation sites predicted by NetOGlyc (Fig. 1C). If these sites were glycosylated, the mass of the peptides would have been altered, and the peptides would not have been identified by MS. Certainly, some post-translational modification of the nCpMuc4 antigen is required for recognition of the host cell receptor, but the nature of these modifications remains a mystery.

Binding of nCpMuc4 to Caco2A cells was also not inhibited by an excess of the CpMuc4 peptide used to raise the neutralizing antibody [21]. This peptide also had no effect on in vitro infection (not shown), suggesting that the CpMuc4 peptide does not encompass the binding epitope or that some post-translational modification or tertiary structure not exhibited by the peptide itself is required for host cell recognition. Inhibition of infection by the anti-CpMuc4 peptide antibody could as well be due to steric hindrance as recognition of the specific epitope involved in binding.

The main obstacle in these studies was extraction of sufficient purified native antigen for experimentation. Generally, between 2 to 6 µg of purified nCpMuc4 could be recovered from 5×10^9 oocysts, but sometimes no detectable antigen could be recovered from particular lot of oocysts. We had previously shown that when the *C. parvum* glycoprotein antigens, gp40 and gp15, are expressed in *T. gondii* the antigens are appropriately post-translationally modified [22,23], and this appeared to be true for CpMuc4 as well. rTgCpMuc4 was nearly identical in size to the native antigen, and the host cell binding kinetics of rTgCpMuc4 closely paralleled nCpMuc4. Additionally, 20 µg of rTgCpMuc4 antigen could be reliably purified from parasites harvested from 10, T150 tissue culture flasks. Unfortunately, biotinylation of rTgCpMuc4 destroyed the binding of the antigen to host cells, and this, in combination with the unknown nature of nCpMuc4 post-translational modifications, prevented a direct comparison of rTgCpMuc4 and nCpMuc4. Other approaches, such as radiolabeling, might be less destructive. Alternatively, comparison of immune responses to nCpMuc4 and rTgCpMuc4 might provide information on the similarity of the two proteins.

While these studies vividly illustrate the difficulty of studying low abundance antigens of *C. parvum*, they also underscore the potential importance of these antigens to host-parasite interactions, and further encourage investigation of nCpMuc4 as potential vaccine candidate. The ability to express a functional antigen in *T. gondii* will enable identification of the host cell receptor and characterization of the molecular interactions between nCpMuc4 and its receptor. Studies such as these will lead to greater insight into *Cryptosporidium* host-parasite interactions, informing the design of new and effective therapeutics to treat cryptosporidiosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

HFF	human foreskin fibroblast
HXGPRT	hypoxanthine-xanthine-guanine phosphoribosyl transferase
IFA	indirect immunofluorescence assay
MWCO	molecular weight cut-off

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Highlights

CpMuc4 is a *C. parvum* antigen involved in parasite host cell invasion

Native CpMuc4 antigen was isolated and characterized

CpMuc4 expressed in *T.gondii* (rTgCpMuc4) is similar to native antigen

Binding of rTgCpMuc4 to host cells parallels binding of native antigen

Post-translational modifications are required for CpMuc4 binding to host cells

Figure 1. Affinity purification of nCpMuc4 with anti-peptide antibody

A. Western blot probed with rabbit anti-CpMuc4 antibody: lane 1: soluble oocyst lysate, pre-purification; lane 2: unbound fraction after incubation with anti-CpMuc4 peptide antibody and Protein G Dynabeads; lane 3: Elutions with CpMuc4 peptide, pooled and concentrated. **B.** Coomassie stain of purified nCpMuc4 (arrow). BSA (*) was identified by N-terminal sequencing. Molecular weight markers in kDa are indicated on the left. **C:** CpMuc4 sequence. Underlines indicate the tryptic peptides identified by LC/MS/MS. Residues predicted to be O- glycosylated are indicated in bold (as predicted by NetOGlyc 4.0: <http://www.cbs.dtu.dk/services/NetOGlyc/>). The predicted N-glycosylation site is in italics (as predicted by ELM: <http://elm.eu.org/>). The arrowhead indicates the predicted signal peptide cleavage site (predicted by SignalP; <http://www.cbs.dtu.dk/services/SignalP/>). The star indicates the first amino acid of the rTgCpMuc4 protein.

Figure 2. CpMuc4 expressed in *T. gondii*

A: IFAs of *T. gondii* tachyzoites, transfected with pHLEM-GRA1CpMuc4 and selected for stable expression, probed with monoclonal antibody to the *T. gondii* surface antigen SAG1 (red) and rabbit anti-CpMuc4 antibody (green). The merged image demonstrates localization of the rTgCpMuc4 protein to the tachyzoite cytoplasm. DIC: differential interference contrast image. Scale bar=10 μ m. **B:** Western blots probed with rabbit anti-CpMuc4 antibody. Lane 1: Affinity-purified rTgCpMuc4, lane 2: affinity purified nCpMuc4, lane 3: affinity purified nCpMuc4; lane 4: purified rEcCpMuc4. Molecular weight markers in kDa are indicated on the left.

Figure 3. CpMuc4 expressed in *T. gondii* mimics binding of the native antigen to Caco2A cells
Caco2A cells were grown to confluence in 96 well plates, fixed with glutaraldehyde, blocked and reacted with native and recombinant CpMuc4 antigens. B-galactosidase was included as a negative control. Samples were run in duplicate and results are representative of two independent experiments. Saturation binding curves for nCpMuc4 and rTgCpMuc4 were generated with Graphpad Prism using the one-site specific binding equation with the minimum x value set to 0.

Figure 4. rEcCpMuc4 and CpMuc4 peptide do not inhibit nCpMuc4 binding to CaCo2A cells
 nCpMuc4 was incubated with fixed CaCo2A cells in the presence of excess rEcCpMuc4 and excess CpMuc4 peptide. Unbound proteins were washed off, and proteins that remained bound to the cells evaluated by western blot probed with rabbit anti-CpMuc4 antibody. The contents of each of the lanes are indicated in the key above the western blot. Arrowhead indicates nCpMuc4 band; arrow indicates rEcCpMuc4. Molecular weight markers in kDa are indicated on the left.

Table 1

Lectins used to detect glycans on nCpMuc4

Lectin	specificity
<i>Helix pomatia</i> (HPA)	! -N-acetyl galactosamine
<i>Artocarpus integrifolia</i> (Jacalin)	! -galactose ; ! -N-acetyl galactosamine
<i>Sophora japonica</i> (SJA)	! -fucose
<i>Glycine max</i> (SBA)	! -N-acetyl galactosamine; ! -N-acetyl galactosamine
<i>Ulex europaeus</i> (UEA)	! -fucose
<i>Maclura pomifera</i> (MPA)	! -galactose ; ! -N-acetyl galactosamine
<i>Arachis hypogaea</i> (PNA)	! -galactose
<i>Canavalia ensiformis</i> (ConA)	! -mannose; ! -glucose; ! -N acetyl glucosamine

Table 2

CpMuc4 peptides identified after in gel digestion with trypsin:

Peptides	Sf score ¹	TIC ²	Ions
GRGNQGGSSNSGSDSGGDTSQFGDPSRPPSPYK	0.97	1.30e04	36/132
PNPFAGVSLSSPRPR	0.97	3.00e03	21/28
LGPFDDGANLKSPR	0.94	4.70e04	17/24
GRGNQGGSSNSGSDSGGDTSQFGDPSRPPSPY	0.93	1.40e03	37/128
PNPFAGVSLSSPRPR	0.93	7.40e02	17/28
GNQGGSSNSGSDSGGDTSQFGDPSRPPSPYK	0.92	5.20e04	31/124
GNQGGSSNSGSDSGGDTSQFGDPSRPPSPYK	0.92	1.40e03	32/124
GRGNQGGSSNSGSDSGGDTSQFGDPSRPPSPY	0.9	1.20e03	35/128
LGPFDDGANLK	0.89	4.10e04	14/18
AGVSLSSPRPR	0.88	2.10e04	14/20
AGVSLSSPRPR	0.87	6.90e03	14/20
LGPFDDGANLK	0.81	5.20e02	12/18
DEESLKGTGDRPDG	0.8	5.00e03	11/26

¹ Sf score: quality of match score [25]

² Total Ion Current

Table 3

CpMuc4 peptides identified after in gel digestion with trypsin, chymotrypsin and pepsin.

Peptides	Sf score ¹	TIC ²	Ions
AGVSLSSPRPR	0.87	2.70e03	15/20
AGVSLSSPRPR	0.87	2.70e03	15/20
DTSQFGDPS	0.68	1.50e04	9/16
DEESLKGTGDRPDG	0.68	8.80e02	11/26
DEESLKGTGDRPDG	0.66	5.10e02	11/26
AGVSLSSPRPR	0.63	1.50e03	14/20
AGVSLSSPRPR	0.63	1.50e03	14/20
DEESLKGTGDRPDG	0.59	8.80e02	11/26
DEESLKGTGDRPDG	0.55	5.10e02	11/26
<i>M*SLIKLSNLGSSGPSNDEESL</i>	0.47	4.40e02	20/88
SSGPSNDEESLKG	0.44	2.00e03	9/24
LSSPRPR	0.44	1.80e04	6/12
SGGDTSQFGDPSRPPSPYKTTTPR	0.42	3.50e02	14/92
SSGPSNDEESLKG	0.42	1.00e03	9/24
DTSQFGDPS	0.41	1.50e04	9/16
<i>M*SLIKLSNLGSSGPSNDEESL</i>	0.32	4.30e02	19/88
PRGRGNQGGSSNSGSDSGGDTSQFGD	0.32	1.80e03	12/50
YGNGKGPHTSNESEDGKLGPF	0.32	4.80e03	9/42
DTSQFGDPS	0.31	8.40e03	7/16
RPRGRGNQGGSSNSGSDSGGDTS	0.31	1.00e05	8/44
<i>MSLIKLSNLGSSGPSNDEESLKGT</i>	0.3	2.90e03	12/100

¹ Sf score: quality of match score [25]

² Total Ion Current