The mucin-like, secretory type-I transmembrane glycoprotein GP900 in the apicomplexan Cryptosporidium parvum is cleaved in the secretory pathway and likely plays a lubrication role

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Research Article

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Abstract

Background

Cryptosporidium parvum is a zoonotic parasite, a member of apicomplexans that possess unique secretory organelles including a rhoptry, micronemes and dense granules that discharge contents during the parasite invasion. The mucin-like glycoprotein GP900 with a single transmembrane domain is one of the immunodominant antigens and micronemal proteins. It was found to be relocated to the surface of excysted sporozoites and shed to form trails by gliding sporozoites, for which the biological process remained unclear. The major aim of this study was to verify whether GP900 was present as a transmembrane protein anchored to the plasma membrane on the surface of sporozoites and whether it was cleaved before being shed from the sporozoites.

Methods

Two anti-GP900 antibodies, a mouse monoclonal antibody (mAb) to the long N-terminal domain (GP900-N) and a rabbit polyclonal antibody (pAb) to the short C-terminal domain (GP900-C), were produced for the detection of intact and cleaved GP900 proteins in sporozoites and other parasite developmental stages by microscopic immunofluorescence assay (IFA) and discharged molecules by ELISA.

Results

Both antibodies recognized the apical region of unexcysted and excysted sporozoites. However, anti-GP900-N (but not anti-GP900-C) also stained the pellicles/surface of excysted sporozoites and trails of gliding sporozoites. Both antibodies stained the intracellularly developing and developed meronts, but not in the macro- and micro-gamonts. Additionally, the epitope recognized by anti-GP900-N (but not anti-GP900-C) was detected in the secretions of excysted sporozoites and intracellular parasites.

Conclusions

GP900 is present in sporozoites and intracellular meronts, but absent in sexual stages. It is stored in micronemes of sporozoites, but enters secretory pathway during excystation and invasion. The short cytoplasmic domain of GP900 is cleaved in the secretory pathway before it reaches to the extracellular space. The molecular features and behaviors imply that GP900 mainly plays a lubrication role.

Background

Cryptosporidium parvum is a zoonotic apicomplexan parasite infecting humans and a wide range of animal species. This parasite is transmitted between human and animal hosts via oral-fecal route by oocysts, an environmental stage of the parasite. A round oocyst (~ 5 μm) contains four banana-like

sporozoites (~ 5×1 μm) that are released in the gastrointestinal tract after being ingested by a host by an excystation process. Released sporozoites invade host enterocytes, residing on top of the host cells in a parasitophorous vacuole (PV) embraced by a host cell-derived membrane termed parasitophorous vacuole membrane (PVM). Before establishment of infection, sporozoites move through the intestinal mucus and glide on the host cell surface. At the infection site, a sporozoite attaches to the host cell that forms PVM engulfing the parasite and the sporozoite is transformed from banana-like shape to a small round trophozoite in the PVM. During the gliding and invasion, a number of proteins are discharged from several specialized secretory organelles called micronemes, rhoptries and dense granules.

One of the currently known molecules secreted from the micronemes is a mucin-like glycoprotein called GP900 (or CpGP900). This glycoprotein is encoded at the locus cgd7_4020 based on the nomenclature for the genome of Iowa-II strain of *C. parvum*. It is defined by an intronless 5814 bp open reading frame (ORF) as a 1937 aa protein, containing a single transmembrane domain (TMD) near the C-terminus. Based on its amino acid composition, the predicted molecular weight of GP900 is 202.6 kDa, whereas its native protein might migrate at a position close to or larger than 900 kDa due to its heavy *O*-linked glycosylation and numerous *N*-linked glycosylation (Fig. 1).

GP900 was first reported 30 years ago as a > 900 kDa glycoprotein as one of the antigens recognizable by hyperimmune bovine colostral (HBC) immunoglobins [1] and a mouse antiserum affinity-purified with the clone S34 in a λgt11 *C. parvum* genomic expression library [2]. It was later defined as a micronemal protein in sporozoites in intact oocysts and merozoites contained in PVM, but distributed to the surface of excysted sporozoites capable of forming trails during gliding and mediating the sporozoite invasion [3]. It was also implied to be one of the molecules tethering the sporozoites with the inner side of the oocyst walls [4]. There were also mass spectrum-based evidence for *O*-linked and *N*-linked glycosylation for GP900, both in relatively simple and unextended forms (also see illustration in Fig. 1) [5, 6].

Overall, the immunodominant antigen GP900 that was implied for involvement of adhesion and invasion of *Cryptosporidium* zoites, while its molecular process during the parasite invasion was yet unknown. Here we provide experimental evidence showing that *C. parvum* GP900 (CpGP900) stored in the sporozoite micronemes is cleaved in the secretory pathway and discharged to the surface and extracellular space of excysted sporozoites in a form lacking the C-terminal cytoplasmic domain. Therefore, secreted CpGP900 is unable to anchor to the sporozoite surface by lacking a transmembrane domain, suggesting that this glycoprotein likely plays a lubrication role during interaction with host cells.

Methods

Parasite materials and general in vitro cultivation protocol

An isolate of *C. parvum* (subtype IIaA17G2R1 based on *gp60* gene) was propagated in calves in-house, and oocysts were purified by sucrose gradient centrifugation as described [7, 8]. Free sporozoites were prepared by an in vitro excystation protocol, in which oocysts were incubated in a serum-free RPMI-1640

medium containing 0.75% taurodeoxycholic acid at 37°C for 45 to 60 min. In vitro culture of *C. parvum* was performed using HCT-8 cell line (human ileocecal colorectal adenocarcinoma; ATCC # CCL-244) to host the parasite growth. HCT-8 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50 units/mL penicillin and 50 μg/mL streptomycin at 37°C in 5% CO_2. For microscopic immunofluorescence assay (IFA), host cells were seeded in 48- or 24-well plates containing poly-L-lysin-treated round glass coverslips and infected with *C. parvum* by inoculation with either free sporozoites or oocysts [9, 10]. The invasion stage was prepared by infecting host cell monolayers (at ~ 60% confluence) with freshly excysted sporozoites for 15 to 30 min [9, 10]. Intracellular stages were prepared by inoculating host cell monolayers with oocysts (viability > 80% based on in vitro excystation rate) at 37°C for 3 h, followed by the removal of uninvaded parasites. After invaded parasites were allowed to grow for various time points between 3 and 72 h post-infection (hpi). For qRT-PCR, host cells were cultured on 96-well plates containing no slides for specified times. All samples prepared above were fixed with formaldehyde, lysed for preparing total RNA or processed as described in following sections.

Antibody production

Two antibodies against CpGP900 were produced in this study: 1) a rabbit polyclonal antibody (pAb) against a short peptide located on the C-terminal cytoplasmic side of the TMD (^{1923}TVVTIERDSSFWNES^{1937}, named as GP900-C), and 2) a mouse monoclonal antibody (mAb) against a peptide on the N-terminal non-cytoplasmic side of the TMD (^{1814}VPGTAAPKKGG^{1825}, named as GP900-N). Both peptides were synthesized by China Peptides Company (Shanghai, China), and conjugated to keyhole limpet hemocyanin (KLH) as described [11]. For pAb production, two rabbits were immunized with KLH-linked GP900-C peptide by subcutaneous injections using a standard 4-injection immunization protocol as described [9, 10, 12]. Serums were collected prior to the first injection and 14 d after the last one. Rabbit antiserums were subjected to affinity-purification with nitrocellulose membrane blotted with peptide GP900-C as described [9, 10, 13]. For mAb production, two Balb/c mice were immunized four times with immunogen (KLH-linked GP900-N; 100 μg with Freund's complete adjuvant for first injection and 50 μg with incomplete adjuvant for next three injections) and a final intravenous injection of 20 μg immunogen containing no adjuvant. Standard protocols were used in hybridoma production [14, 15]. A clone GP900 AB5 was selected after three round of ELISA screening and used for producing mouse ascites.

Western blot analysis

Parasite oocysts were disrupted by five freeze/thaw cycles between liquid nitrogen and ice, and lysed with a radioimmunoprecipitation assay (RIPA) buffer containing 1% Triton X-100 and protease inhibitor cocktail for eukaryotes (Sigma Aldrich, St. Louis, MO) on ice overnight, followed by additional 1 h incubation at room temperature. Lysates equivalent to 1.0×10^7 sporozoites/lane were heated with reducing sample buffer for 5 min at 95°C, fractionated with 6% SDS-PAGE and transferred onto nitrocellulose membranes. The blots were blocked in 5% skim milk-TBST, probed with affinity-purified anti-GP900-C pAb (1:100 dilution in blocking buffer) or anti-GP900-N mAb (1:50 dilution in blocking

buffer) for 1 h. After three washes with blocking buffer, blots were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (i.e., goat anti-rabbit IgG or goat anti-mouse IgG; Invitrogen, Waltham, MA, USA), and visualized using an enhanced chemiluminescence reagent (Beyotime Biotechnology, Shanghai, China) according manufacturers' instructions. All procedures were conducted at room temperature unless specified.

Immunofluorescence microscopic assay (IFA)

Intact oocysts after disruption by freeze/thaw and excysted sporozoites of *C. parvum* were fixed with 4% formaldehyde for 30 min, washed with PBS and applied onto glass slides coated with poly-L-lysine. After air-dry for 1 to 2 h, samples were permeabilized with 0.2% Triton X-100 for 5 min and treated in a blocking solution containing 3% BSA in PBS for 1 h. In some experiments, permeabilization step was omitted for antibody-labeling of proteins on the outside of the fixed sporozoites. In gliding trail experiments, excysted sporozoites in RPMI-1640 medium were placed onto poly-L-lysine coated slides for 20 min at 37°C, followed by the same fixation and permeabilization procedures. Invading sporozoites and intracellular meronts on host cell monolayers were prepared as described in the first section of Materials and Methods, followed by fixation, permeabilization and blocking as described above.

For immunolabeling, samples were incubated with affinity-purified anti-GP900-C pAb or anti-GP900-N mAb in blocking buffer for 1 h, followed by three washes in blocking buffer and incubation for 1 h with one of the following secondary antibodies: Alexa Fluor 488-labeled goat anti-rabbit-IgG antibody, Alexa Fluor 488-labeled goat anti-mouse-IgG antibody (Thermo Scientific, West Palm Beach, FL; 1:2,000 dilution in PBS). Some specimens were also co-stained with rhodamine-phalloidin to visualize host cell F-actin. Samples were then stained for nuclei with 4,6-diamidino-2-phenylindole (DAPI) (1.0 μg/mL). There were three washes with PBS between steps (5 min each). All procedures were performed at room temperature. Slides were examined under an Olympus BX53 research fluorescence microscope. Images were capture with an Olympus DP72 camera and stored in TIFF format. The signal levels of images were linearly adjusted with Adobe Photoshop 2021 without local manipulations and presented with Adobe Illustrator 2021.

qRT-PCR Analysis of GP900 Gene Transcripts

Total RNA samples were isolated from oocysts, excysted sporozoites and intracellular parasites cultured in HCT-8 cells at 3, 6, 12, 24, 48 and 72 hpi using an iScript qRT-PCR sample preparation reagent (Bio-Rad Labs, Hercules, CA). The relative transcript level of *CpGP900* were detected by a SYBR Green-based qRT-PCR using HiScript II One-Step qRT-PCR SYBR Green Kit (Vazyme Biotech Co., Nanjing, China) with primer 5'-ATC TAT TCC TCC AAG CGT ACC A-3' and 5'-CAT TAT TGG GTT CAA GTC ACC A-3'. For quality control, the transcripts of previously characterized lactate dehydrogenase (*CpLDH*) and elongation factor 1α (*CpEF1a*) genes were also detected using primers as described [8, 16, 17]. The transcript of *C. parvum* 18S rRNA (Cp18S) was detected for normalization as described [8, 18].

qRT-PCR were performed in 20 μL volume containing 0.2 μM of each primer, 1.0 μL One Step SYBR enzyme mix, 10 μL SYBR Green mix, and 0.4 μL ROX reference dye 1 (50×), 0.2 ng of total RNA isolated

from oocysts/sporozoites or 15 ng total RNA of intracellular parasites. Reactions started with 50°C for 3 min to synthesize cDNA, 95°C for 30 s to inactivate reverse transcriptase, and 40 cycles at 95°C for 10 s and 60°C for 30 s to produce amplicons. Each samples included ≥ 2 biological replicates and ≥ 2 technical replicates in qRT-PCR reactions. Relative transcript levels were calculated using an empirical $2^{-\Delta\Delta CT}$ formula as described [8, 18, 19].

ELISA detection for the secretion of CpGP900

The secretion of CpGP900 proteins was evaluated by ELISA in excysted sporozoites, during invasion and intracellular developmental stages between 3–6 hpi, 10–12 hpi and 20–24 hpi. Free sporozoites were prepared by in vitro excystation as described above. A portion of the sporozoites ($1x10^7$) immediately after excystation were washed three times with PBS by centrifugation and resuspended in serum-free RPMI-1640 medium. After incubation at 37°C for 1 h, supernatants were collected by centrifugation (12,000 g for 5 min). The remaining sporozoites were used to infect HCT-8 monolayers at ~ 90% confluence in 48-well plates ($5x10^5$/well) for collecting following samples. Invasion stage was preparing by inoculating excysted sporozoites (after three washes after excystation) in serum-free RPMI-1640 medium for 3 h, followed by collection of supernatants (i.e., 0–3 hpi stage). Other intracellular stages were prepared by culturing parasites in serum-containing complete medium that was replaced by serum-free medium for specified time periods (i.e., 3–6 hpi, 10–12 hpi and 20–24 hpi). Supernatants were collected from the wells at the end of each time period, followed by replacement of complete medium and continuous culture to the next time period. Positive specimen control used crude extracts prepared from excysted sporozoites ($1x10^7$) by three freeze/thaw cycles and collection of supernatants by centrifugation (12,000 g for 5 min).

To detect secreted CpGP900, supernatants prepared above were mixed with ELISA coating buffer (0.05 M carbonate-bicarbonate, pH 9.6) and added to 96-well ELISA plates (50 µL/well), followed by incubation overnight at 4°C. Plates were rinsed with PBS and blocked with 3% BSA in PBS (100 µL/well) at 37°C for 1 h. After three washes with 0.05% Tween-20 in PBS (PBS-T buffer), plates were incubated with affinity-purified pAb to GP900-C (1:5 dilution) or mAb to GP900-N (1:5 dilution) in PBS-T buffer (50 µL/well) 37°C for 1 h. A mouse antiserum (1:10 dilution) raised against total proteins in *C. parvum* sporozoites was used as a positive control for secreted proteins. After three washes with PBS-T buffer, plates were incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (ImmunoWay Biotechnology Co., Plano, Texas, USA; 1:10000 dilution) or goat anti-mouse IgG(H + L) (ImmunoWay Biotechnology Co., 1:10000 dilution) secondary antibodies, followed by color development with p-nitrophenyl phosphate (Sigma-Aldrich, St. Louis, MO, USA) as instructed by the manufacturers. Optical density at 405 nm (OD_{405}) was read in a multifunctional microplate reader (BioTek, Vermont, USA). Experiments were performed three times independently and each include at least two biological replicates and two technical replicates.

Results

Molecular and domain features of the single-pass type I membrane GP900

The glycoprotein GP900 (1937 aa) is a single-pass membrane protein consisting of an N-terminal signal peptide (SP; positions 1–26 aa) and a single transmembrane domain (TMD; 1863–1887 aa) near the C-terminus (Fig. 1). The presence of an SP and a TMD indicates that GP900 is a type I membrane protein in the secretory pathway, in which GP900 is translated on the endoplasmic reticulum (ER) with N-terminal domain inserted into the ER lumen for initial glycosylation and further glycosylation in Golgi body. The SP is cleaved in the ER after the completion of translation. It predicts that the long N-terminal domain (~ 1837 aa) and very short C-terminal domain (~ 50 aa) are non-cytoplasmic and cytoplasmic, respectively. GP900 as a secretory glycoprotein was also confirmed by its presence in the secretory organelle micronemes in sporozoites and merozoites by IFA and IEM in earlier studies [1–3], and in this study as well (see later sections for detail).

GP900 contains a large number of Thr (n = 503; 26%) and Ser (n = 155; 8%) residues, including two large Thr-rich stretches, that are heavily *O*-glycosylated, and an average number of Asn residues (n = 89; 4.6%) that are partially *N*-glycosylated (Fig. 1, marked in green or blue) [5, 6]. GP900 is an intrinsic disorder protein by possessing 10 intrinsic disorder regions across the entire protein (Fig. 1, marked in brown), suggesting its overall structure flexibility. GP900 also possesses 11 Cys residues (Fig. 1, marked in red), all located on the N-terminal half of the protein, suggesting some intramolecular (and potentially intermolecular) cross-links by disulfide bonds to increase the stability of the N-terminal half of the protein.

The transcript of *GP900* gene could be detected in oocysts, free sporozoites and intracellular developmental stages (Fig. 2). The transcript levels were relatively high in oocysts and sporozoites when normalized with Cp18S transcript, but varied dramatically in intracellular stages. The lowest levels were observed shortly after invasion (i.e., 3 and 6 hpi; representing early development of meronts), elevated to the highest at 12 hpi (representing the maturation and formation of merozoites in the first generation of merogony), and then steadily declined from 24 hpi to 48 hpi and 72 hpi (representing much less synchronized second and third generations of merogony and gametogenesis).

The C-terminal cytoplasmic domain of GP900 is cleaved in the secretory pathway before reaching to cell surface in *C. parvum* sporozoites

GP900 was known to be “stored in micronemes prior to appearance on the surface of invasive forms” and discharged to form trails during the gliding of sporozoites [20, 21]. We were interested in confirming the relocation of GP900 from micronemes to cell surface and testing whether this single-pass membrane protein was present on the sporozoite surface as a transmembrane protein in the cytoplasm membrane. Therefore, we raised two antibodies to distinguish the C-terminal cytoplasmic domain (rabbit anti-GP900-

C pAb) from the N-terminal non-cytoplasmic domain (mouse anti-GP900-N mAb) (Fig. 1; dark red bars). Both anti-GP900-C pAb (affinity-purified) and anti-GP900-N mAb (prepared in the mouse ascites) recognized a single high molecular weight band above the 250 kDa marker in western blot analysis (Fig. 3). The immunoblotting data was similar to those observed in earlier studies [1–3], confirming the specificity of the two antibodies.

IFA using anti-GP900-C antibody confirmed the protein location in the apical region packed with micronemes in the sporozoites before and after excystation (Fig. 4A, 4B). In this assay, immunolabeling intact oocysts was obstructed by the impermeability of oocyst walls, but resolved by rupturing oocysts with freeze/thaw cycles to allow the access of antibodies to sporozoites [9]. The anti-GP900-N antibody also chiefly labeled the apical region in sporozoites before excystation (as indicated by the presence on one side of the nuclei) (Fig. 4C). However, in excysted sporozoites, anti-GP900-N antibody not only strongly labeled the apical region, but also along the pellicle with weaker but clear signals (Fig. 4D). The labeling of anti-GP900-N antibody on the surface of sporozoites were confirmed by IFA of formaldehyde-fixed, but non-permeabilized sporozoites (Fig. 5A), in which slightly weaker but clear fluorescent signals were observable on the surface of sporozoites, but no signals were detected inside of the sporozoites. For direct comparison, the fluorescent signals from permeabilized sporozoites were detected along the pellicles and inside of the sporozoites that were processed in parallel under the same conditions (Fig. 5B). Furthermore, in the gliding sporozoites, anti-GP900-N antibody again labeled sporozoite pellicles and the gliding trails of some sporozoites (Fig. 5C), whereas anti-GP900-C antibody only labeled the apical region of sporozoites (Fig. 5D).

To give a short summary of the IFA data, GP900 present in the micronemes of the sporozoite apical region contained both C-terminal cytoplasmic and N-terminal non-cytoplasmic domains (i.e., full length GP900, minus signal peptide in theory), whereas the GP900 protein distributed along the sporozoite surface or pellicles contained only the N-terminal non-cytoplasmic domain. The gliding sporozoites also discharged GP900 that lacking the C-terminal cytoplasmic domain. These observations concluded that the C-terminal domain of GP900 was cleaved in the secretory pathway before it reached to the cell surface.

GP900 is present in asexual developmental stages, but absent in sexual stages

During the sporozoite invasion of host cells, anti-GP900-C antibody again labeled apical region in the long sporozoites (early invasion), but became two dense spots in short sporozoites (later invasion) or two semi-circles surrounding the newly formed trophozoites (Fig. 6A). Anti-GP900-N antibody also labeled apical region (more strongly) plus the pellicles in long sporozoites, and became more diffused in short sporozoites and newly formed trophozoites (Fig. 6B). During *C. parvum* intracellular development, immunostaining by anti-GP900-C antibody was apparent (Fig. 7A, green), showing signals surrounding

nuclei in small meronts with one nucleus (Fig. 7A, upper two panels) or strong signals surrounding the large developing meronts with multiple nuclei (Fig. 7A, lower left panel). In mature meronts in which banana-like merozoites were formed, fluorescent signals became concentrated on one side of the nuclei that implied apical distributions in merozoites (Fig. 7A, lower right panel).

Based on co-staining with PVM using a mouse polyclonal antibody against total *C. parvum* proteins (Fig. 7A, red), the immunostaining by anti-GP900-C antibody was confined to the parasites within the PVM, rather than on the PVM. In IFA using anti-GP900-N antibody (Fig. 7B), uneven signals were observed in mature meronts, implying distributions on the apical region of merozoites, but the signals appeared weak in trophozoites and developing meronts. However, we also observed high background/non-specific signals from host cells using anti-GP900-N antibody that could not be resolved after multiple attempts of changing experimental conditions. In fact, anti-GP900-C antibody also produced relatively weak signals in developing meronts (vs. stronger signal in developed meronts), but it produced low background/non-specific signals in host cells, allowing us to increase exposure time to visualize the specific signals.

While GP900 was observed in all asexual stages including sporozoites and meronts, it was undetectable in the sexual stages of *C. parvum*. Neither anti-GP900-C nor anti-GP900-N antibodies produced signals in sexual stages, and both antibodies only produced close-to-the-background signals in macrogamonts and microgamonts (vs. clear signals using the anti-total protein antibody) (Fig. 8).

GP900 is released into extracellular space by sporozoites and intracellular parasites

The lower signals from anti-GP900-N antibody in intracellular parasites were also implied by the fact that a significant amount of processed/cleaved GP900 was secreted/discharged to the extracellular space (i.e., into the culture medium) as determined by ELISA (Fig. 9). In this assay, anti-GP900-N antibody (Fig. 9 orange bars), but not anti-GP900-C antibody (green bars), detected secreted GP900 in the medium after incubation with excysted sporozoites (37°C for 1 h), and in the serum-free medium collected from the invasion stage (i.e., 0–3 hpi) and selected intracellular developmental stages (i.e., 0–3, 3–6, 10–12 and 20–24 hpi). Two positive reagent controls showed expected results: 1) in the specimen control, both anti-GP900-N (orange bars) and anti-GP900-C (green bars) antibodies detected GP900 from the supernatant of sporozoite lysate prepared by freeze/thaw; and 2) in the antibody control, the antibody raised against total parasite proteins detected antigens in all samples (gray bars). The OD values were relatively low for the intracellular samples (Fig. 9A), but still notable, particularly when individual datasets were normalized with the OD values obtained using anti-total protein antibody (Fig. 9B). Normalization with antiserum against total parasite proteins was less perfect, as the antiserum might not recognize all *C. parvum* proteins equally and different parasite stages might also secrete proteins unequally in specified time periods. However, this normalization gave a rough, but sufficient comparison on the relative levels of secreted GP900 between various stages.

Taking together all the ELISA and IFA data, we might build a working model for the biological process of GP900 in sporozoites (Fig. 10): 1) In intact oocysts, this single-pass transmembrane glycoprotein is

stored in the micronemes of sporozoites (Fig. 10A-B); 2) it starts to enter the secretory pathway during/after the excystation (Fig. 10C, steps 1), in which the short C-terminal domain is cleaved at the TMD site (step 2) and the secretory vesicles cross the inner membrane complex (IMC) consisting of flatted plates and fused with plasma membrane (PM) (step 3); and 3) the long N-terminal domain is released into the extracellular space (step 4). Because of the general adhesive property for a glycoprotein and the presence of charged amino acids, some discharged GP900 molecules retained on the sporozoites surface. The cleavage is presumably carried out at the TMD by one of the three *C. parvum* rhomboid peptidases (CpROMs) (Fig. 10D), in which CpROM1 has been recently confirmed to be a micronemal protein as well [10]. In fact, GP900 contained an intramembrane sequence with a high identity to that of known cleavage site of *Toxoplasma gondii* micronemal proteins (i.e., AA|GG in GP900 vs. IA|GG in TgMIC2 and TgMIC6) (Fig. 10D).

Discussion

The mucin-like GP900 was discovered ~ 30 years ago as an immunodominant protein that was present in micronemes, but relocated to the surface of sporozoites and shed from the surface during gliding motility [1–3]. Little new knowledge was gained on the biology of GP900 in the past two decades, though. In this study, we were initially interested in knowing how GP900 was shed from the surface of gliding sporozoites. We thought that this type I membrane protein was anchored to the sporozoite plasma membrane via the TMD, but cleaved from the surface based on its presence on gliding trails. Therefore, we raised two antibodies specific to the long N-terminal and short C-terminal domains separated by the single TMD (i.e., anti-GP900-N and anti-GP900-C antibodies). Surprisingly however, while both antibodies labeled the apical region of sporozoites by IFA, only anti-GP900-N antibody, but not anti-GP900-C antibody, labeled the sporozoite surface and gliding trails. This observation indicates that GP900 protein distributed along the sporozoite pellicles or surface lacks the C-terminal domain. Because both antibodies labeled apical region of sporozoites in intact oocysts before excystation, the cleavage of the C-terminal domain should take place after GP900 enters the secretory pathway but before it reaches the surface of sporozoites during and after the excystation.

Similar to classic mucins, GP900 is heavily glycosylated, but both *O*-glycans and *N*-glycans are in relatively simple forms. For *O*-glycans, which is the major type of glycans in mucins and GP900, *C. parvum* lacks enzymes to further extend *O*-GalNAc core, similar to the engineered “SimpleCell” lines that only expresses truncated *O*-GalNAc [5]. In the case *N*-glycans, they form only a single arm that is barely processed in the ER or Golgi [6]. Therefore, GP900 contains much less polysaccharides in comparison to classic mucins, making it less expandable in the water-containing extracellular environment. Also because *O*-glycans are unextended, GP900 lacks terminal negatively charged sialic acid as seen in intestinal mucins, although it may be slightly negatively charged based on the theoretical isoelectric point (pI = 4.62). This physiochemical property is favorable for the diffusion of released GP900 molecules at the infection sites by avoiding electrostatic repulsion from the highly negatively charged intestinal mucins. In the gliding trail IFA assay, the diffusion of GP900 molecules into the medium also explains that the trails were detected only from some (but not all) gliding sporozoites (Fig. 5C).

GP900 was previously reported to be one of the glycoproteins that tether sporozoites to the inner surface of the oocyst wall by IFA and IEM assays [4]. However, in this study, both anti-GP900-C and anti-GP900-N antibodies failed to label the inner side of or around the oocyst walls in ruptured oocysts (Fig. 4). We noticed that in the study by Chatterjee and colleagues used two monoclonal antibodies that were specific to an *N*-linked epitope (mAb 4G12) and *O*-linked GalNAc (mAb 4E9), rather than specific to GP900 only. Therefore, the antigens recognized by 4G12 and 4E9 antibodies could be other glycoprotein containing *N*- or *O*-glycans, rather than GP900. In other words, there is a presence of glycoproteins, but not GP900, that tether sporozoites and oocyst walls. This notion is also supported by the absence of adhesive domains in GP900 necessary for binding/tethering molecules (Fig. 1), such as APPLE, TSP, SUSHI and EGF domains commonly presence in adhesin proteins [22, 23].

Based on the physiological properties (mucin-like, weakly charged and weakly adhesive), the presence in sporozoites and developed merozoites, and the discharge of cleaved molecules to the surface of sporozoites and extracellular space, we may speculate that GP900 mainly plays a lubrication role during the gliding, invasion and intracellular development.

Conclusions

Using IFA with two antibodies to the long N-terminal non-cytoplasmic domain (anti-GP900-N) and the short C-terminal cytoplasmic domain (anti-GP900-C), we confirm that the mucin-like glycoprotein GP900 in *C. parvum* was stored in the micronemes of sporozoites and merozoites, and discovered that its short cytoplasmic domain was cleaved in the secretory pathway before the protein was released to the extracellular space of sporozoites during/after excystation. The molecular properties and the behaviors of GP900 in different developmental stages also implies that this protein mainly plays a lubrication role.

Declarations

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Authors' contributions

GZ and JY designed and supervised the study; XL, DQ XG, YZ and MW performed the experiments; XL, DW, JY and GZ analyzed the data; XL, JY and DW wrote the manuscript draft; GZ revised and finalized the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval

The study was approved by the Ethics Committee of Jilin University Institute of Zoonosis (Animal use protocol # IZ-2019-084). Animals were handled in China's accordance with the Animal Ethics Procedures

and Guidelines.

Consent for publication

Not applicable.

Competing interests

The authors declare that they no competing interests.

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Figures

Figure 1

Architecture of the mucin-like *Cryptosporidium parvum* GP900 protein. It contains an N-terminal signal peptide (SP) for targeting the protein to endoplasmic reticulum and a transmembrane domain (TMD) near the C-terminus to separate the long non-cytoplasmic domain and the short cytoplasmic domain. There are 10 intrinsic disorder regions with various lengths across the protein (brown), numerous sites for *O*-glycosylation including two long Thr-rich regions (green), a number of sites for *N*-glycosylation (blue), and 15 Cys residues (red). Sites recognized by the mouse anti-GP900-N monoclonal antibody (mAb) and the rabbit anti-GP900-polyclonal antibody (pAb) are also mark (purple).

Figure 2

Relative level of transcripts of *C. parvum* GP900 (*CpGP900*), lactate dehydrogenase (*CpLDH*) and elongation factor 1α (*CpEF1α*) genes in oocysts, excysted sporozoites and intracellular parasites developed for various times as determined by qRT-PCR. The relative levels were normalized with those of *Cp18S* transcript. *CpLDH* and *CpEF1α* were included and detected in parallel for comparison and quality control.

Figure 3

Western blot detection of native GP900 protein in the crude extracts of oocysts using affinity-purified anti-GP900-C polyclonal antibody (**A**) and anti-GP900-N monoclonal antibody (**B**). Note that the band recognized each antibody was above the upper most band (250 kDa) in molecular markers (M). DIC, differential interference contract microscopy.

Figure 4

Immunofluorescence assay (IFA) detection of GP900 protein (green) in ruptured oocysts (**A** and **C**) and excysted sporozoites (**B** and **D**) using affinity-purified polyclonal anti-GP900-C (**A-B**) and monoclonal anti-GP900-N (**C-D**) antibodies. Nuclei (blue) were counterstained with 4,6-diamidino-2-phenylindole (DAPI). Ruptured oocysts were prepared by three cycles of freeze-and-thaw of intact oocysts in 4% formaldehyde. DIC, differential interference contract microscopy.

Figure 5

Immunofluorescence assay (IFA) detection of GP900 protein (green) in excysted sporozoites. (**A-B**) IFA detection of GP900 protein on the external surface of the sporozoites that were formaldehyde-fixed but unpermeabilized (**A**) in comparison with permeabilized sporozoites (**B**) using anti-GP900-N antibody. For fair comparison, both samples were processed in parallel under the same experimental conditions. Note that weak, but clear when over-exposed, signals were produced in unpermeabilized sporozoites. (**C-D**) IFA detection of GP900 protein on the trails of gliding sporozoites using anti-GP900-N (**C**) and anti-GP900-C (**D**) antibodies, in which anti-GP900-N antibody stained surface of sporozoites and some gliding trails (**C**) while anti-GP900-C antibody only stained the apical region of the gliding sporozoites (**D**). In all panels, nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). DIC, differential interference contract microscopy.

Figure 6

Immunofluorescence assay (IFA) detection of GP900 protein (green) in invading sporozoites using anti-GP900-C (**A**) and anti-GP900-N (**B**) antibodies. Host cells F-actin was stained with phalloidin–rhodamine (red), and nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) (blue). DIC, differential interference contract microscopy.

Figure 7

Immunofluorescence assay (IFA) detection of GP900 protein (green) in intracellularly developing parasite, including trophozoites, developing and mature meronts using anti-GP900-C (**A**) and anti-GP900-N (**B**) antibodies. In panel **A**, the parasite were labeled with a mouse clonal antibody against total parasite proteins that produced strong signals in parasitophorous vacuole membrane (PVM) (red). In both panels,

nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). DIC, differential interference contract microscopy.

Figure 8

Immunofluorescence assay (IFA) detection of GP900 protein (green) in microgamonts and macrogamonts (sexual stages) using anti-GP900-C (**A**) and anti-GP900-N (**B**) antibodies. In panel **A**, the parasite were labeled with a mouse clonal antibody against total parasite proteins that produced strong signals in parasitophorous vacuole membrane (PVM) (red). In both panels, nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). DIC, differential interference contract microscopy.

Figure 9

ELISA detection of secreted GP900 protein from various parasite stages. **A**) Relative levels of secreted GP900 from excysted sporozoites (i.e., serum-free RPMI-1640 medium after incubation with free sporozoites after the removal of excystation medium) and intracellular parasite cultured for specified times (i.e., serum-free medium placed between 0-3, 3-6, 10-12 and 20-24 h post-infection (hpi)) using antibodies against GP900-C epitope (green), GP900-N epitope (orange) and total parasite proteins (gray). **B**) The same optical density datasets from panel **A** after normalization with those derived from antibody against total parasite proteins.

Figure 10

A working model for the biological process of GP900 in sporozoites: GP900 protein (**A**) is stored in the secretory organelle micronemes in sporozoites in oocysts before excystation (**B**). After excystation and during invasion, GP900 in the microneme vesicles enter secretory pathway (**C**, step 1). Before the secretory vesicles reach to the plasma membrane, the short C-terminal cytoplasmic domain of GP900 is cleaved (step 2). The vesicles reach to and fused with the plasma membrane (step 3) and cleaved GP900 molecules were discharged to the extracellular space (step 4). Some secreted GP900 molecules adhere to the surface of plasma membrane by general adhesive property of the glycoprotein, while other molecules shed into the surrounding microenvironment. The transmembrane domain of GP900 contains conserved amino acids (purple) near the non-cytoplasmic side for intramembrane cleavage by rhomboid peptidases (**D**). DG, dense granules; IMC, inner membrane complex; MN, microneme; Nuc, nucleus; PM, plasma membrane; TMD, transmembrane domain.