Cryptosporidium Parvum

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Cryptosporidium parvum is a zoonotic intracellular protozoan responsible for the diarrheal illness cryptosporidiosis in humans and animals. Although a number of zoite surface proteins are known to be expressed during, and believed to be involved in, attachment and invasion of host cells, the molecular mechanisms by which C. parvum invades the host epithelial cells are not well understood. In the present study, we investigated the gene expression patterns, protein localization in developmental stages in culture, and in vitro neutralization characteristics of Cpgp40/15 and Cpgp40. Indirect immunoflfluorescence assay showed that Cpgp40/15 is associated with the parasitophorous vacuole membrane (PVM) during intracellular development. Both anti-gp40/15 and anti-gp40 antibodies demonstrated the ability to neutralize C. parvum infection in vitro. Further studies are needed to fully understand the specifific role and functional mechanism of Cpgp40/15 (or gp40/15 complex) in the invasion of the host or in the PVM and to determine the feasibility of gp40/15 as a vaccine candidate for cryptosporidiosis in vivo.

Keywords: Cryptosporidium parvum ; Cpgp40/15 ; PVM ; neutralization ; vaccine

1. Ethics Statement

The animal handling and experimental procedures were carried out in compliance with recommendations of the Guide for the Care and Use of Laboratory Animals of the Ministry of Health, China. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Henan Agricultural University on July 10, 2015 (authorization number (IACUC-henau-20150710).

2. Parasite and Cell Lines

C. parvum (Iowa isolate) oocysts were purchased from Waterborne, Inc. (New Orleans, LA, USA) and stored in phosphate-buffered saline (PBS) at 4 °C for up to 3 months (from harvest) before use. Before experiments, oocysts were treated with 10% Clorox on ice for 10 min and washed three times with sterile PBS. Free sporozoites were prepared by incubating oocysts in PBS containing 0.25% trypsin and 0.75% taurodeoxycholic acid at 37 °C for 2 h. Human ileocecal adenocarcinoma (HCT-8) cells (American Type Culture Collection, Manassas, VA, USA) were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 U/mL streptomycin at 37 °C in a humidified 5% CO_2 incubator. For in vitro experiments, HCT-8 cells were transferred to 12-well cell culture plates and monolayers grown to 80%–90% confluence. *C. parvum* oocysts were added into the cell culture at a parasite:host cell ratio of 1:5 (i.e., 2×10^5 oocysts/well). After incubation at 37 °C for 3 h that allowed sporozoites invade host cells, uninvaded parasites were removed by a medium exchange. Intracellular parasites were allowed to grow for specified times before subsequent experiments including RNA isolation for gene expression analysis or fixation for immunofluorescence staining.

3. Cpgp40/15 and Cpgp40 Cloning, Expression, and Purification

The following two fragments were amplified by PCR from *C. parvum* (Iowa) genomic DNA with the following primers (the added restriction sites are underlined): a 903-bp fragment encoding 294 amino acids (corresponding to the entire Cpgp40/15 ORF (open reading frame), minus the putative signal peptide), forward, 5'-CGCGAATTCGATGTTCCTGTTGAGGGCTC-3'; reverse, 5'-CGCGTCGACCAACACGAATAAGGCTGC-3'. A 588-bp fragment encoding 190 amino acids (corresponding to Cpgp40), forward, 5'-CGCGAATTCGATGTTCCTGTTGAGGGCTC-3'; reverse, 5'-CGCGTCGACCTCTGAGAGTGATCTTCTTG-3'. PCR amplification was performed under the following conditions: denaturation at 95 °C for 5 min, 35 cycles of amplification at 95 °C for 45 s, 57 °C for 45 s, and 72 °C for 1 min; and a final extension at 72 °C for 10 min. PCR products were purified using a TIANgel Midi Purification Kit (TIANGEN Biotech, Beijing, China), digested with EcoRI and SalI restriction enzymes (New England Biolabs, Beijing, China), and inserted into the expression vector pGEX-4T-1 (Novagen, Madison, WI, USA). *Escherichia coli* DH5α cells (TIANGEN

Biotech, Beijing, China) were transformed with the ligation products and grown on Luria–Bertani (LB) agar plates with 50 μg/mL ampicillin, with positive colonies being identified by PCR and sequencing. *E. coli* Rosetta (DE3) cells (TIANGEN Biotech) were transformed with the recombinant plasmids and cultured in LB medium supplemented with 50 μg/mL ampicillin. The induction of *Cpgp40/15* and *Cpgp40* expression was performed by adding 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 37 °C for 3 h. The expression levels and solubility of the target proteins were evaluated by using SDS-PAGE with Coomassie blue G-250 staining.

For the purification of Cpgp40/15 and Cpgp40, cultured *E. coli* were collected by centrifugation, re-suspended in PBS buffer, and disrupted by sonication on ice. The lysate was centrifuged and the supernatant was filtered through a 0.45 μm cellulose acetate membrane filter (Millipore, Billerica, MA, USA) and loaded onto Glutathione Sepharose 4B beads (GE Healthcare, Pittsburgh, USA) at 4 °C and 90 rpm for 3 h. After washing the beads with six volumes of PBS, Cpgp40/15 and Cpgp40 were eluted from the beads with elution buffer (50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0). The purified proteins were examined by SDS-PAGE on a 10% gel.

4. Preparation of Cpgp40/15 and Cpgp40 Polyclonal Antibodies

Polyclonal antibodies against Cpgp40/15 and Cpgp40 were raised in pathogen-free rabbits by Sangon Biotech (Shanghai, China). Primary immunization was conducted on days 1 and 21 using 300 μg of purified Cpgp40/15 and Cpgp40 protein emulsified in an equal volume of Freund's complete adjuvant. Immunized animals received boost immunizations four times every seven days with 150 μg of Cpgp40/15 and Cpgp40 protein in Freund's incomplete adjuvant. Seven days after the final immunization, rabbit sera were collected, and the polyclonal IgG antibodies were purified from the immune sera using protein A Sepharose affinity chromatography. The titer and specificity of the antibodies were evaluated using an enzyme-linked immunosorbent assay (ELISA) and Western blot, respectively.

5. Western Blot Analysis of Native Cpgp40/15 and Cpgp40

For Western blot analysis of the native Cpgp40/15 and Cpgp40, oocysts treated with 10% Clorox were suspended in PBS buffer containing 0.75% taurodeoxycholic acid and 0.25% trypsin and incubated at 37 °C for 3 h. The released sporozoites were collected by centrifugation and resuspended in cell lysis buffer containing 1% protease inhibitor cocktail (Solarbio, Beijing, China). Similarly, HCT-8 cells (≈2 × 10^6) were lysed in the same way. The proteins (from ≈2 × 10^7 sporozoites/lane and ≈5 × 10^5 HCT-8 cells/lane) were separated by SDS-PAGE and transferred onto a polyvinylidene fluoride (PVDF) membrane using a semi-dry electro-blotting apparatus (Bio-Rad, Hercules, CA, USA) running at 300 mA for 2 h. After blocking with PBST containing 5% bovine serum albumin (BSA) at room temperature (RT) for 1 h, the membrane was incubated overnight with anti-Cpgp40/15 antibodies (≈1.3 μg/mL), anti-Cpgp40 antibodies (≈1.3 μg/mL), or pre-immune serum (1:500). The following day, the PVDF membrane was washed three times with PBST and incubated at RT with 1:2000 horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (H+L) (Proteintech, Wuhan, China) for 1 h. Finally, the membrane was washed three times with PBST and reactive protein bands in the membrane were detected using the Immobilon Crescendo Western HRP substrate (Merck Millipore, MA, USA) and analyzed with an Amersham Imager 680 (GE, CT, USA).

6. Indirect Immunofluorescence Microscopy

Sporozoites resuspended in PBS were dried onto microscope slides, while the intracellular stages of *C. parvum* in HCT-8 cell were grown on coverslips for 9, 12, and 18 h. The slides and coverslips were fixed at RT for 20 min with 4% paraformaldehyde. After three washes in PBS, the fixed cells were permeabilized with 0.5% Triton X-100 in PBS for 15 min, washed three times with PBS, blocked with 5% BSA in PBS (BSA-PBS) at RT for 30 min, and incubated overnight with anti-Cpgp40/15 and anti-Cpgp40 antibodies (≈1.3 μg/mL) in 5% BSA-PBS, respectively. In addition, the slides and coverslips treated as above and incubated overnight with pre-immune serum served as controls. After three washes in PBS, the cells were incubated with Alexa Fluor® 594-conjugated goat anti-rabbit IgG (Bioss, Beijing, China) in BSA-PBS at 1:500 for 1 h. After three washes with PBS, the cells were counter stained with the nuclear stain 4′, 6-diamidino-2-phenylindole (DAPI, Yeasen, Shanghai, China). After another three washes with PBS, the slides and coverslips were mounted with No-Fade Mounting Medium (Yeasen, Shanghai, China) and examined by differential interference contrast (DIC) and fluorescence microscopy using a LSM 710 laser confocal microscope (Zeiss, Jena, Germany).

7. Examination of Cpgp40/15 Expression by qPCR

The relative expression levels of the *Cpgp40/15* gene in intracellular parasites in HCT-8 cultures at 0–48 h (3, 6, 9, 12, 24, and 48 h post-infection) was evaluated by qPCR. The expression of the 18S rRNA gene was determined in parallel for data normalization [1]. Using HiPure Total RNA Plus Kits (Magen, Guangzhou, China), total RNA was extracted from *C. parvum*-infected HCT-8 cells at each culture point. Then, first-strand cDNA was synthesized using the PrimeScript™ RT Reagent Kit with gDNA Eraser (TaKaRa, Dalian, China). qPCR was performed in a 25 µL reaction volume containing 2 µL (approximately 100 ng) of cDNA, 12.5 µL of 2× SYBR1 Premix Ex TaqTM II (Tli RNaseH Plus, TaKaRa), 0.5 µL each of the forward and reverse primers (10 µM), and 7 µL of deionized water in a CFX384™ Real-Time PCR system (Bio-Rad, Hercules, CA, USA). The primers used included 5'-GATTTGTTTGCCTTTACCCT-3' and 5'-CCAAGTCTCCGTTCTCATTC-3' for the *Cpgp40/15* gene and 5'- TAGAGATTGGAGGTTGTTCCT-3' and 5'- CTCCAC CAACTAAGAACGGCC-3' for 18S rRNA. The relative expression of the *Cpgp40/15* gene was calculated using the $2^{-\Delta\Delta CT}$ method [2]. The data are presented as the means ± standard error of the mean (SEM).

8. In Vitro Neutralization of Sporozoite Invasion

The effect of polyclonal anti-Cpgp40/15 and anti-Cpgp40 antibodies on *C. parvum* infection of HCT-8 cells were examined using an in vitro neutralization assay. Briefly, HCT-8 cells were grown in 12-well plates to 80%–90% confluence and maintained in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 U/mL streptomycin at 37 °C in a humidified 5% CO_2 incubator. Oocysts were treated with 10% Clorox on ice for 10 min and washed three times with cold sterile PBS. For neutralization assays, 2×10^5 oocysts were incubated with different dilutions of antibodies or pre-immune serum in infection medium in HCT-8 cell culture at 37 °C for 2 h. Based on results of preliminary evaluations, 1:50 (≈13 µg), 1:100 (≈6.5 µg), and 1:500 (≈1.3 µg) dilutions of antibodies were used in neutralization assays. After 3 h incubation, free sporozoites were washed off and incubated for an additional 24 h. The method for assessment of antibodies against *C. parvum* infection of HCT-8 cells in vitro was based on the quantitative real-time reverse transcription-PCR (qRT-PCR) technique, as described in Cai et al. [1].

References

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