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Original article

Differential expression of *Cryptosporidium parvum* genes encoding sporozoite surface antigens in infected HCT-8 host cells

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Abstract

Intracellular replication of *Cryptosporidium parvum* (Apicomplexa) involves the generation of several asexual and sexual forms of the parasite. During the stage conversions, complex mechanisms lead to differential structural and functional properties of the parasite. These require a well tuned gene transcription machinery. For the first time the gene expression of four surface proteins of *C. parvum* sporozoites, CP15, CP17, P23, and GP900 were analysed in parallel by reverse transcription polymerase chain reaction. In addition, CP17 and P23 antigens were detected in infected host cells by immunofluorescence using antisera raised against recombinant forms of the proteins. The results show that expression of each gene follows a unique time schedule during intracellular development, suggesting that the functions of these proteins during the life cycle are not restricted to the invasive stages.

Keywords: Antigens; Cell culture; *Cryptosporidium parvum*; Gene expression; Surface proteins

1. Introduction

Cryptosporidium parvum is an intracellular protozoan parasite that infects primarily the epithelial cells of the small intestine. Infection can lead to watery diarrhoea as the major symptom. In immunocompetent individuals the disease is self-limiting. However, in patients with an impaired immune system the course of the infection can become chronic and life-threatening as seen in patients with AIDS. *Cryptosporidium parvum* belongs to a group of parasites termed the *Apicomplexa*. These organisms are characterised by a complex of secretory organelles (i.e. micronemes, rhoptries and dense granules) at the apical end of the invasive stages, the zoites. After release from the oocyst, sporozoites have to find, attach and invade the target cell. This is a parasite-driven process involving both parasite surface proteins and secretions from the apical organelles. After internalisation, the secretory organelles disappear while the trophozoite stage is formed. Intracellular development of the meront leads to the generation of merozoites which are structurally and functionally similar to the sporozoites. Merozoites are released from meronts to start further rounds of host cell invasion, asexual reproduction, and release. De novo biosynthesis of apical complex organelles and other components of the zoites is required in each round of merogony.

In vitro cultivation systems for *C. parvum* are valuable tools to study the intracellular replication of the parasite. Here we analysed the expression of the genes encoding four *C. parvum* zoite stage antigens, CP15, CP17, P23, and GP900 in HCT-8 host cells over 98 h post infection (p.i.). These parasite proteins are involved in the invasion and/or the host immune response to infection. CP15, CP17 and P23 regularly lead to an antibody response in the infected host and specific immunoglobulin can serve as a serologic marker of infection [1]. P23 contains neutralisation-sensitive epitopes [2] and appears, together with the CP15 antigen, to be the most promising

Abbreviations: IFAT, immunofluorescence antibody test; Mab, monoclonal antibody; p.i., post infection; RT-PCR, reverse transcription polymerase chain reaction.

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candidate for vaccine development [3]. The multi-domain mucin-like GP900 inhibits sporozoite invasion in MDCK host cells [4]. This glycoprotein can also stimulate *C. parvum*-specific proliferative immune responses of mesenteric lymph node cells in C57BL/6J mice [5].

Using RT-PCR and immunofluorescence assays we have found substantial differences in the extent and time course of expression within the group of genes encoding the zoite surface proteins.

2. Materials and methods

2.1. Parasites

Oocysts of *C. parvum* (Iowa strain) passaged in new born calves were obtained from commercial sources (Pleasant Hill Farm, Troy, ID and Bunch Grass Farm, Drury, ID). According to the suppliers, oocysts were purified by sucrose density centrifugation, washed and resuspended in PBS. Parasites were stored at 4 °C in the presence of 1000 U ml^{-1} penicillin and 1000 μg ml^{-1} streptomycin. Before the experiments, oocysts were treated with 1:20 diluted commercial bleach (conc. 3.8% sodium hypochlorite; 5 min on ice) in order to surface sterilise the parasites, and then washed three times in sterile PBS. In some experiments, oocysts were excysted for 2 h at 37 °C in RPMI medium (Gibco) containing 0.75% Na-taurocholate before RNA preparation.

2.2. Host cells

The ileocecal adenocarcinoma cell line HCT-8 was purchased from the ATCC (#CCL244). Cells were passaged in maintenance medium at 37 °C in 6% CO_2/94% humidified air incubator as described [6].

2.3. In vitro development of C. parvum

Cell culture flasks (Nunclon surface 75 cm^2, Nunc, Wiesbaden, Germany) were seeded with $0.5-1 \times 10^7$ HCT-8 cells 18 h before adding *C. parvum* oocysts. Cell culture maintenance medium was replaced by growth medium with supplements as described [7] including $1-2 \times 10^7$ *C. parvum* oocysts. Oocysts were allowed to excyst in situ for 2 h at 37 °C and then removed and replaced by growth medium. Cultures were incubated for additional 6, 12, 24, 48, 72, and 96 h and fed with additional growth medium to maintain pH values and to supply nutrients over the time of cultivation. Uninfected host cells from each time point served as negative controls.

Starting at 2 h after addition of oocysts cultures were washed three times with PBS-K^+(10 mM Na_2HPO_4, 10 mM KH_2PO_4, 150 mM NaCl, 15 mM KCl, pH 7.4). Cells were harvested after treatment with trypsin-EDTA (10 min at 37 °C; Sigma) by centrifugation and washed in PBS-K^+. Cell pellets were stored at −70 °C until further processing.

2.4. DNA and RNA preparation

At various time points cells were harvested from culture flasks and processed. Chromosomal DNA and total RNA were prepared using commercial kits (DNeasy Tissue Kit and RNeasy Mini Kit, respectively; Qiagen). For RNA preparation cell pellets were homogenised (QIAshredder, Qiagen) and additionally treated with DNase (Qiagen). In addition to infected host cell cultures, RNA was also prepared from an excystation mixture containing free sporozoites and intact, unexcysted oocysts.

2.5. cDNA synthesis, primers and PCR conditions

Synthesis of cDNA was performed with 2 μg of total RNA using a commercial kit (Omniscript RT Kit, Qiagen). The Primer3 software [8] was used to design primers specific for *C. parvum* CP15, CP17, P23, GP900, COWP and human GAPDH (Table 1). For uniform amplification profiles primers were selected to have a G/C content of approximately 50%, a T_m of 60 °C and a product size of approximately 200 bp. Consequently, all PCR amplifications could be run in parallel in a tube-controlled thermocycler (omnigene, Hybaid, Heidelberg, Germany). PCR and RT-PCR of the *C. parvum* 18S rRNA was performed at 55 °C. The amplifications were carried out with 100 ng chromosomal DNA or 1 μl cDNA (equivalent to 100 ng of total RNA) from infected host cells. When RT-PCR from freshly excysted sporozoites was performed, cDNA amounts equivalent to 1 and 10 ng total RNA were used. Twenty picomoles of each forward and reverse primer, dNTPs (100 mM each), 5 μl of 10× PCR buffer and 2.5 units of HotStarTaq DNA polymerase (Qiagen) were added to the template in a final volume of 50 μl. The polymerase was activated for 15 min at 95 °C. A total of 25–35 cycles were performed with 1 min denaturing at 94 °C, 1 min annealing at 60 °C (55 °C for 18S rRNA), and 1 min elongation at 72 °C. After the last cycle a 10 min elongation step at 72 °C was added. Complementary DNA preparations from uninfected HCT-8 cell cultures at each time point were used as negative controls in RT-PCR analysis. In order to check for genomic DNA contamination, amplification of DNase-treated RNA preparations from infected host cells were performed, which yielded no PCR products (not shown). Of the total PCR reaction, 10 μl were analysed on a 1.5% agarose gel. Amplification products were stained with ethidium bromide after agarose gel electrophoresis and recorded with a digital camera (Kodac documentation and analysis system 120). For better presentation of weaker bands, black and white photographs are presented in the inverted mode.

2.6. Recombinant proteins

For generation of antisera, recombinant forms of *C. parvum* surface antigens CP17 and P23 were expressed in *E. coli* and purified. CP17 [9] also called gp15/45/60 [10] is a highly polymorphic antigen of *C. parvum* zoite stages. On the basis of GenBank entry sequence Acc. No. AF114166 we designed