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Molecular & Biochemical Parasitology 140 (2005) 43–53
MOLECULAR & BIOCHEMICAL PARASITOLOGY

Eimeria tenella microneme protein EtMIC3: identification, localisation and role in host cell infection☆

M. Labbé[a,*], P. de Venevelles[a], F. Girard-Misguich[b], C. Bourdieu[a], A. Guillaume[b], P. Péry[a]

[a] *Laboratoire de Virologie et Immunologie Moléculaires INRA, 78352 Jouy-en-Josas, France*
[b] *Université de Versailles, Saint-Quentin-en-Yvelines, Département Biologie, 78035 Versailles, France*

Received 25 August 2004; received in revised form 26 November 2004; accepted 8 December 2004
Available online 29 December 2004

Abstract

The gene coding for *Eimeria tenella* protein EtMIC3 was cloned by screening a sporozoite cDNA library with two independent monoclonal antibodies raised against the oocyst stage. The deduced sequence of EtMIC3 is 988 amino acids long. The protein presents seven repeats in tandem, with four highly conserved internal repeats and three more divergent external repeats. Each repeat is characterised by a tyrosine kinase phosphorylation site, WRCY, and a reminiscent motif of the thrombospondin1 (TSP1)-type I domain, CXXXCG. The protein EtMIC3 is localised at the apex of free parasite stages. It is not detected in the early intracellular parasite stage but is synthesised in mature schizonts. Secretion of the protein is induced when sporozoites are incubated in complete medium at 41 °C. Strangely enough, the two independent mAb that allow cloning of EtMIC3 interfere with parasitic growth in different ways. One is able to inhibit parasite invasion whereas the other inhibits development. Expression and localisation of the protein EtMIC3 are consistent with a protein involved in the invasion process as is expected for a microneme protein.

Keywords: *Eimeria*; Microneme; Adhesive domain; Thrombospondin; Invasion; Schizogony; Monoclonal antibodies

1. Introduction

Apicomplexan parasites—*Plasmodium*, *Babesia*, *Cryptosporidium*, *Isospora*, *Cyclospora*, *Sarcocystis*, *Eimeria* and *Toxoplasma*—are well known for their invasion processes via the unique apical complex present at the apex of invasive forms. The initial interaction of zoites of apicomplexan parasites with cells is probably a random collision and presumably involves reversible interactions between proteins on the parasite surface and the host cell. Invasion begins when the apical end of the zoite attaches itself to the host cell surface, forming a tight adhesion zone that moves backwards as the zoite penetrates into the nascent vacuole [1]. Gliding motility, involved both in locomotion and in host cell invasion [2,3], is a substrate-dependent locomotion, which implicates secretion of apical vesicules—the micronemes and the rhoptries. It occurs without any change in the cell shape and results from backward capping of parasite surface molecules that are attached to the host membrane receptors. The connection of these surface molecules with the sub-pellicular actin–myosin network of the parasite, which acts as the motor, involves an aldolase [4,5]. After initial contact between the parasite and the host, micronemes are first expelled [6]. This is followed by sequential secretion from rhoptries and dense granules that leads to the formation and maintenance of the parasitophorous vacuole that surrounds the parasite in the host cytoplasm (see [7–9] for additional information concerning invasion by *Apicomplexa*). The invasion process is completed within 5–10 s. Several authors have shown that apicomplexan parasites migrate through the host cells before they replicate

☆ *Note:* Nucleotide sequence data reported in this paper are available in the GenBank™ database under the accession numbers AY512381 and AY512382.

* Corresponding author. Tel.: +33 1 34 65 26 18; fax: +33 1 34 65 26 21.
E-mail address: marie.labbe@jouy.inra.fr (M. Labbé).

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doi:10.1016/j.molbiopara.2004.12.002

in an appropriate host cell [10–12]. After replication, parasites then evade from the host cell after an asexual replication and reinvade another cell. Little information is available about egress but it probably involves the same mechanisms as entry [13].

Analysis of the sequence of apicomplexan parasite microneme proteins reveals common features [14,15]. Many of the microneme proteins characterised so far contain adhesive domains, including TSP1-type I domains, integrin insertion (I) domains, epidermal growth factor (EGF) domains, Apple (A-) domain of Von Willebrand factor, also known as I domain, and Apple domains. The presence of these domains indicates that these microneme proteins play key roles in attachment and invasion. The *Plasmodium* sporozoite-specific transmembrane protein known as thrombospondin-related anonymous protein (TRAP) is stored within micronemes [16], becomes surface-exposed at the anterior tips of the zoite and is released onto the substrate during gliding locomotion [9]. Genetic alteration of the TRAP cytoplasmic tail abrogates sporozoite gliding and loss of invasion is observed after mutations in two adhesive modules of the TRAP ectodomain [17,18]. Since these modules are also present in other apicomplexan microneme proteins of the TRAP family, such as MIC2 in *Neospora* and *Toxoplasma* and MIC1 in *Eimeria*, they may account for the capacity of the parasite to enter many cell types. Following the secretion of microneme content, proteins can either stay attached to the parasite membrane or be secreted. For example, during active penetration into the host cell, TgMIC2 occupies the moving junction and parasite surface, where it translocates rearwards before being released from the posterior end as the host membrane closes around the parasite [19]. Microneme proteins are often cleaved (for more information, see [20]). These processing events are essential for invasion [21]. Proteases MPP1 and MPP2 can cleave the C- and N-terminal extremities of *Toxoplasma gondii* MIC2, respectively [7].

Various microneme proteins have been described in *Eimeria tenella*. EtMIC1 is a member of the TRAP protein family with one A-domain of Von Willebrand, five TSP-1 modules and well-conserved transmembrane and C-terminal regions [22]. EtMIC2 is secreted and implicated in the gliding of sporozoites of *E. tenella* [23]. EtMIC4 is also a member of the TRAP protein family that contains 31 EGF modules, 12 TSP-1 modules and a highly acidic proline and glycine-rich region in its extracellular region. EtMIC4 is constitutively expressed on the surface of sporozoites and merozoites [24]. EtMIC4 is highly immunogenic since chicken mucosal antibodies are essentially directed against this protein [25]. EtMIC5 is rich in cysteine motifs that have striking similarities to the adhesive Apple domains of blood coagulation factor XI [26]. EtMIC5 is secreted into the culture supernatant during parasite infection of MDBK cells. Expression of proteins EtMIC1, 2, 4 and 5 is detected at various times during the sporulation process. Messenger RNA for all of these microneme proteins were detected 10–12 h earlier than proteins, suggesting that expression of microneme protein is regulated at the transcriptional and translational levels [27].

In this paper, we describe the *E. tenella* microneme protein (EtMIC3) whose cDNA was isolated from an expression cDNA library of sporozoites by two different mAb. The EtMIC3 sequence shows seven repeats and each repeat possesses a reminiscent motif of the TSP1-type I domain. A search for similarities showed that the predicted protein has identity with domains of the microneme protein NcMIC1 of *Neospora caninum* and TgMIC1 of *T. gondii*. We also showed that its expression is regulated during schizogony and that specific mAb could inhibit the in vitro growth of *E. tenella*.

2. Materials and methods

2.1. Parasite and in vitro culture of E. tenella

E. tenella was maintained by passage through coccidia-free two-week-old chickens. Unsporulated oocysts were obtained from the caecal contents of chickens seven days post-infection. They were purified and allowed to sporulate using standard procedures [28]. Sporozoites were purified on a cotton column as described [28].

CS3 cells [29] were used for infection inhibition assays. These cells were cultured in F12 medium (Gibco, BRL), supplemented with 1% fœtal calf serum (FCS). Sporozoites were added to semi-confluent monolayers of CS3 cells at a ratio of three sporozoites per cell. Infected monolayers were incubated at 41 °C. Cells were washed 20 h p.i. and fresh medium was added.

CEV-1 cells (a generous gift from Dr. D. Brake [30]) were used for immunofluorescence experiments. CEV-1 cells were infected with one sporozoite per cell for 64 h in Medium 199 (Sigma) containing 5% FCS at 41 °C.

2.2. Immunoscreening of a cDNA library and cloning of a cDNA coding for EtMIC3

Two hybridoma that expressed mAb A6 and N8 were obtained following an immunisation of mice with a sporulated oocyst lysate [31]. A cDNA library specific to the sporozoite stage [25] was screened with these two monoclonal antibodies as previously described [32]. After two rounds of plaque purification, recombinant pBluescript plasmids of several positive clones were excised.

2.3. Sequencing and analysis of clones coding for EtMIC3

The two extremities of the cDNA of each clone were sequenced with T3 and T7 primers using the dideoxy chain termination technique (Dye Deoxy Terminator kit, USB) and loaded on a fluorescent 373A automated cDNA sequencer