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EnP1 and EnP2, two proteins associated with the *Encephalitozoon cuniculi* endospore, the chitin-rich inner layer of the microsporidian spore wall

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Abstract

Microsporidia are obligate intracellular parasites forming environmentally resistant spores that harbour a rigid cell wall. This wall comprises an outer layer or exospore and a chitin-rich inner layer or endospore. So far, only a chitin deacetylase-like protein has been shown to localize to the *Encephalitozoon cuniculi* endospore and either one or two proteins have been clearly assigned to the exospore in two *Encephalitozoon* species: SWP1 in *E. cuniculi*, SWP1 and SWP2 in *Encephalitozoon intestinalis*. Here, we report the identification of two new spore wall proteins in *E. cuniculi*, EnP1 and EnP2, the genes of which are both located on chromosome I (ECU01_0820 and ECU01_1270, respectively) and have no known homologue. Detected by immunoscreening of an *E. cuniculi* cDNA library, *enp1* is characterized by small-sized 5′ and 3′ untranslated regions and is highly expressed throughout the whole intracellular cycle. The encoded basic 40 kDa antigen displays a high proportion of cysteine residues, arguing for a significant role of disulfide bridges in spore wall assembly. EnP2 is a 22 kDa serine-rich protein that is predicted to be *O*-glycosylated and glycosylated phosphatidyl inositol-anchored. Although having been identified by mass spectrometry of a dithiothreitol-soluble fraction, this protein contains only two cysteine residues. Mouse polyclonal antibodies were raised against EnP1 and EnP2 recombinant proteins produced in *Escherichia coli* Our immunolocalisation data indicate that EnP1 and EnP2 are targeted to the cell surface as early as the onset of sporogony and are finally associated with the chitin-rich layer of the wall in mature spores.

Keywords: Microsporidia; Encephalitozoon cuniculi; Spore wall; Endospore; Cysteine; Immunolocalization

1. Introduction

Microsporidia are obligate intracellular parasites found in a wide variety of animal hosts (Weiss, 2001). Species from several genera, including *Encephalitozoon*, have been recognized as opportunistic human pathogens, some of these causing severe diarrhoea in AIDS patients (Bryan and Schwartz, 1999). However, serological studies indicated that microsporidiosis may also occur in immunocompetent hosts (van Gool et al., 1997; 2004). Microsporidia have 70S ribosomes and lack typical eukaryotic organelles such as mitochondria and peroxysomes. This likely results from a highly regressive evolution, given that phylogenetic analyses support the conception that microsporidia are closely related to fungi (Keeling, 2003; Thomarat et al., 2004).

The extracellular infective stage of microsporidia is the spore which is surrounded by a rigid cell wall and contains a remarkable invasion apparatus. A structure coiled within the spore, called the polar tube, can be suddenly extruded then used to transfer the sporoplasm into the cytoplasm of a host cell. The first part of the intracellular development corresponds to a proliferative phase (merogony), during which meronts divide by binary or multiple fission. The second part called sporogony begins when meronts transform into sporonts that are characterized by the deposition of an electron-dense material on the plasma membrane. Sporonts divide into sporoblasts in which the formation of the thick wall is associated with the differentiation of the invasion apparatus. In *Encephalitozoon* species, all the development takes place inside a parasitophorous vacuole. After completion of the maturation of sporoblasts

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into spores, the rupture of host cells may lead to the release of spores in the environment.

The spore wall consists of two major layers: (i) an electron-dense outer layer of 25–30 nm, the exospore, which is principally proteinaceous, and (ii) an electron-lucent inner layer of 30–35 nm, the endospore, which contains chitin and proteins (Bigliardi et al., 1996). This wall makes the spore resistant to various environmental stresses and its aqueous permeability may play a role in the control of the polar tube discharge activation (Frixione et al., 1997).

The microsporidian spore wall has been shown to be extremely resistant to dissociation in the absence of thiol-reducing agents such as dithiothreitol (DTT) or 2-mercaptoethanol (Keohane et al., 1996; Hayman et al., 2001). Few data about the protein components of this structure are available, although a variety of antibodies directed against microsporidia were frequently found to be specific to spore wall antigens (Beckers et al., 1996; Enriquez et al., 1997; Delbac et al., 1998a; Lujan et al., 1998; Prigneau et al., 2000). The sequences of either one or two exospore proteins have been identified in two *Encephalitozoon* species: spore wall protein (SWP)1 and SWP2 in *Encephalitozoon intestinalis* (Hayman et al., 2001) and only SWP1 in *Encephalitozoon cuniculi* (Bohne et al., 2000). These are cysteine-rich polypeptides with similar N-terminal domains and with repeating amino acid units at their C-terminus. The localization of a potential chitin deacetylase at the endospore-plasma membrane interface has been recently demonstrated in *E. cuniculi* (Brosson et al., 2005). Taking advantage of the availability of the full sequence of the *E. cuniculi* nuclear genome (2.9 Mbp; Katinka et al., 2001), we report here the characterization of two different *Encephalitozoon* Endospore Proteins (EnPs) after either immunoscreening of a cDNA library for EnP1 or mass spectrometry analysis of a DTT-soluble protein extract for EnP2.

2. Materials and methods

2.1. Microsporidian spore production and purification

Encephalitozoon cuniculi was grown in vitro on MDCK (Madin-Darby canine kidney) or HFF (human foreskin fibroblast) cells, in minimum essential medium (MEM) supplemented with 5% FCS and 2 mM glutamine at 37 °C in a 5% CO_2 atmosphere. Spore-containing supernatants were collected every 72 h. Spores were harvested (18,000×*g*, 2 min), washed and stored in PBS at 4 °C.

2.2. Antibody production

Polyclonal antibodies to *Escherichia coli* expressed recombinant proteins were produced in BALB/c mice from SDS-PAGE separated protein bands. Protein bands were excised from Coomassie blue-stained gel and crushed in PBS with a Potter apparatus. Mice were then injected intraperitoneally with samples homogenized with FCA for the first injection and Freund incomplete adjuvant for the next injections (at days +14, +21 and +28). Sera were collected 1 week after the last injection and stored at −20 °C.

The monoclonal antibody Ec102 was raised against a 55 kDa band separated by SDS-PAGE (Delbac et al., 1998a) and was shown to be directed against both *E. cuniculi* polar tube protein (PTP)1 (Delbac et al.,1998b) and PTP2 (Delbac et al., 2001).

2.3. SDS-PAGE and Western blotting

Total *E. cuniculi* proteins were extracted in a lysis buffer containing 2.5% SDS and 100 mM DTT. Spores were disrupted by 10 steps of freezing and thawing in liquid nitrogen, followed by sonications (10×30 s on ice) and boiled for 15 min. After centrifugation for 5 min at 18,000×*g*, the residual insoluble pellet was incubated for a further 72 h in 200 mM DTT, 2.5% SDS and boiled for 15 min. Protein samples were then analyzed by SDS-PAGE. After electrophoresis, proteins were stained with Coomassie blue or transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore). For immunological detection, membranes were saturated in PBS, 5% milk and incubated for 3 h with an appropriate dilution of mouse antibodies (1:100–1:1,000). After washing, membranes were reacted with horseradish peroxidase-conjugated goat anti-mouse IgG, A and M (Sigma) and developed with the ECL Western blot detection kit (Amersham).

2.4. Indirect immunofluorescence assay

Encephalitozoon cuniculi infected-HFF cells grown on glass slides were fixed with 4% paraformaldehyde, 0.05% glutaraldehyde for 20 min at room temperature then washed in PBS, 0.1 M glycine. Cells were permeabilized with PBS, 0.5% Triton X-100 and blocked with 5% skim milk in PBS. Slides were incubated for 1 h with mouse sera diluted in PBS, 0.1% Triton X-100, washed and incubated further 1 h with a 1:1000 dilution of Alexa 488 conjugated goat anti-mouse IgG (Molecular Probes) or a 1:400 dilution of TRITC-conjugated goat anti-mouse IgG (Sigma). Preparations were examined with a DMR Leica epifluorescence microscope.

2.5. Transmission electron microscopy immunolabeling

Intracellular parasites grown on MDCK cells were fixed with 4% paraformaldehyde, 0.05% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2. Cells were then scraped off, dehydrated and embedded in Unicryl resin. Ultra-thin sections were blocked with PBS, 0.1% BSA, incubated for 3 h with mouse sera diluted in PBS, 0.25% BSA, washed and incubated further 1 h with a 1:100 dilution of goat anti-mouse IgG gold-conjugated (Sigma). After staining with 4% uranyl acetate, preparations were examined with a JEOL 1200X transmission electron microscope.