EnP1, a Microsporidian Spore Wall Protein That Enables Spores To Adhere to and Infect Host Cells In Vitro^{7}

Timothy R. Southern, Carrie E. Jolly, Melissa E. Lester, and J. Russell Hayman^{*}
Department of Microbiology, James H. Quillen College of Medicine, East Tennessee State University, Johnson City, Tennessee 37614

Received 9 April 2007/Accepted 26 May 2007

Microsporidia are spore-forming fungal pathogens that require the intracellular environment of host cells for propagation. We have shown that spores of the genus Encephalitozoon adhere to host cell surface glycosaminoglycans (GAGs) in vitro and that this adherence serves to modulate the infection process. In this study, a spore wall protein (EnP1; Encephalitozoon cuniculi ECU01_0820) from E. cuniculi and Encephalitozoon intestinalis is found to interact with the host cell surface. Analysis of the amino acid sequence reveals multiple heparin-binding motifs, which are known to interact with extracellular matrices. Both recombinant EnP1 protein and purified EnP1 antibody inhibit spore adherence, resulting in decreased host cell infection. Furthermore, when the N-terminal heparin-binding motif is deleted by site-directed mutagenesis, inhibition of adherence is ablated. Our transmission immunoelectron microscopy reveals that EnP1 is embedded in the microsporidial endospore and exospore and is found in high abundance in the polar sac/anchoring disk region, an area from which the evert ing polar tube is released. Finally, by using a host cell binding assay, EnP1 is shown to bind host cell surfaces but not to those that lack surface GAGs. Collectively, these data show that given its expression in both the endospore and the exospore, EnP1 is a microsporidian cell wall protein that may function both in a structural capacity and in modulating in vitro host cell adherence and infection.

Microsporidia are obligate intracellular spore-forming protists that are classified as divergent fungal pathogens. During the AIDS epidemic, microsporidia were identified as being a causative agent of severe diarrhea that plagued many human immunodeficiency virus-positive individuals (5). However, microsporidiosis is not completely limited to the immunocompromised, as there are many reports of immunocompetent persons contracting the disease (19). More recently, it has been suggested that microsporidia could be classified as zoonotic organisms since they have been found in both wild and domesticated animals as well as animals often kept as pets, including birds, dogs, and rabbits (20).

Due to the water-borne nature of microsporidia, transmission usually occurs via the fecal-oral route. Therefore, the initial site of infection is intestinal epithelial cells. Unlike any other fungal agents, microsporidia gain entry into host cells through a series of complex, but poorly understood, mechanisms that culminate in the release of a coiled filament (or tube) from within the spore, which penetrates the host cell cytoplasmic membrane. The infectious material, called sporoplasm, travels from the spore through the tube into the host cell cytoplasm where replication occurs, safe from external immune responses. The destruction of the intestinal epithelium results in intestinal crypt hyperplasia, decreased surface area, and malabsorption, which leads to watery diarrhea.

Prior to polar tube release, it is thought that a spore receives a stimulus that triggers activation. At this time, the nature of the stimulus is not known, but there are many varied recipes for spor germination and polar tube release in vitro (15). The general consensus is that following activation, an influx of ions into the spore results in the displacement of calcium and the activation of a trehalase conversion enzyme that breaks down trehalose into glucose and metabolites (14). This conversion results in an osmotic event, the swelling of the internal structures, and the buildup of significant turgor pressure. The pressure is released by the rupture of the spore at the apex and the subsequent eversion of the polar tube (14).

Our previous studies have shown that the in vitro adherence of Encephalitozoon spores to host cells precedes activation and is mediated by host cell surface glycosaminoglycans (GAGs) (12). When adherence is inhibited by exogenous sulfated glycosaminoglycans or by certain divalent cations, host cell infection significantly decreases or increases, respectively (12, 21). These data shape our current hypothesis that microsporidian spore adherence is an integral part of activation and host cell infection. It is speculated, based on these in vitro data, that the ability of microsporidia to infect a wide range of hosts and tissues may correlate with their ability to utilize multiple GAGs for adherence (12).

This study was designed to identify possible ligands in the spore wall that are involved in the adherence process. The spore wall is a rigid structure composed of an electron-dense outer layer that contains protein and an electron-lucent structural layer containing protein and chitinous material (23). These layers are separated from the cell by a plasma membrane. Although the spore wall affords the spore environmental protection, only a few proteins that reside in it have been identified. To date, two proteins are known to be in the exospore region, and three have been found within the endospore region in members of the family Encephalitozoonidae. The exospore proteins (SWP1 and SWP2) have repetitive amino...
acid sequence units of unknown functions (1, 10). One of the endospore proteins (EnP2 or SWP3) is a 20- to 22-kDa protein predicted to be O glycosylated and glycosylphosphatidylinositol anchored to the plasma membrane (17, 24). Another endospore protein (EnP1) is cysteine rich and is postulated to be involved in spore wall assembly by disulfide bridging (17). The third protein, a putative chitin deacetylase (EcCDA), is present in two isoforms (33 and 55 kDa) and is associated with the plasma membrane of developing spores (2). In this study, we show that the EnP1 protein is not limited to the endospore but is also found on the anchoring disk complex and on the exposed exosporial surface. In addition, we show that this protein acts as a ligand for spore adherence to host cells.

**MATERIALS AND METHODS**

**Microsporidia and host cell cultivation.** African green monkey kidney cells (Vero; ATCC CCL-81) and rabbit kidney cells (RK-13; ATCC CCL-37) were used for the cultivation of microsporidian spores. Adherent cells were maintained in Dulbecco’s modified Eagle’s medium (BioWhittaker, Walkersville, MD) supplemented with l-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), amphotericin B (0.25 µg/ml), and 2% fetal bovine serum (Bio-Whittaker) in 5% CO₂ at 37°C. Microsporidian spore propagation and purification were performed as previously described (11).

**Transmission IEM.** Confluent RK-13 monolayers grown in 75-cm² flasks were infected with E. cuniculi or Encephalitozoon intestinalis spores and were maintained until numerous infected cells were visible by light microscopy. Heavily infected monolayers were prepared for immunoelectron microscopy (IEM) as previously described (9). Briefly, the infected monolayers were fixed with 2% paraformaldehyde and 0.05% glutaraldehyde, scraped from the flask, and en-robod in 3% SeaKem agar. The cell pellet was subsequently dehydrated in methanol. The pellet was then imbedded and photopolymerized in Lowicryl K4M resin. Ultrathin sections were applied to grids, and immunolabeling was performed. A 1:10 dilution of the primary anti-EnP1 antibody was used, followed by a 1:200 dilution of AuroProbe EM 15-nm gold-labeled anti-rabbit secondary antibody (GE Healthcare, Piscataway, NJ). After counterstaining with 5% uranyl acetate, the sections were viewed using a Tecnai 10 (FEI) transmission electron microscope. By using these antibody concentrations, very little background staining of host cell cytoplasm was observed. Control sections were treated with the secondary antibody only to show no significant background staining.

**Site-directed mutagenesis.** Heparin-binding motifs were sequentially deleted from the parent EcEnP1 gene that was previously cloned into the pET21a vector by site-directed mutagenesis kit according to the manufacturer’s recommendations (Stratagene, Cedar Creek, TX). The primer sets used were designed to delete heparin-binding motifs 1 (5’-GATGGATTGACGACGATCTGTTGGCCGACTACGGATGGATCACTGACATCTC-3’ and 5’-GATGGATTGACGACGATCTGTTGGCCGACTACGGATGGAAGCT-3’), HBM2 (5’-TACACGAAAACTTCCACACACGAGCTCAG-3’ and 5’-AACACGGAAGCTCAGAGGTCTGTTGGAAGCTCAGGTT-3’), and an N-terminal portion of the EcEnP1 gene (5’-TACACGAAAACTTCCACACACGAGCTCAG-3’ and 5’-TTCCACATTGGAAGCTCAGGTTGGAAGCTCAGGTT-3’). Each mut-ated construct was expressed and purified as described above.

**Spore adherence assays.** Microsporidian spore adherence and infection were measured as previously described (12). Either Vero or RK-13 cells were used as host cells and were seeded onto glass coverslips (18 mm) in 12-well plates and grown to confluence. To test adherence, recombinant proteins (1 µg/ml) were added to each well and incubated at 37°C for 2 h in the presence or absence of EcEnP1 antibodies. To test infection, recombinant proteins (1 µg/ml) were added to each well and incubated for 2 h in the presence or absence of EcEnP1 antibodies. To measure host cell infection, the monolayers of host cells with bound spores were returned to culture for an additional 36 h to allow the microsporidial spores to develop. Following incubation, the coverslips were stained with 0.01% UVex for 10 min at room temperature. UV microscopy was used to evaluate infection at x400 magnification. The results are expressed as the percentage of host cells infected per field of magnification. Statistical differences were determined using the Student t test. For both adherence and infection assays, experiments were repeated at least three times.

**RESULTS**

**Identification of a microsporidian adherence protein, EnP1.** We have shown that E. intestinalis and E. cuniculi spore adherence to host cells is governed by host cell surface GAGs (12). To confirm that spore constituents are involved in the
process, proteins from *E. cuniculi* spores were used as an inhibitor in spore adherence assays. With increasing amounts of total spore protein, *E. cuniculi* adherence is impeded in a dose-dependent manner, whereas approximately 10-fold more total *E. coli* protein had no effect (Fig. 1). These data indicate that the spore protein lysate contains proteins that may serve as adherence ligands. To identify the ligand(s), *E. cuniculi* protein lysate was biotinylated and reacted with intact host cell monolayers in a host cell binding assay by using a Western blotting approach. Unbound protein was removed by washing, and the monolayers were prepared for Western analysis. While the control (unlabeled host cell protein without biotinylated spore proteins) showed nonspecific reactions of host cell proteins with streptavidin, one biotinylated *E. cuniculi* spore protein of approximately 40 kDa was clearly detected (Fig. 2). While the control lanes include biotin-labeled TCA-precipitated (TCA ppt) *E. cuniculi* spore protein and host cell protein without the addition of biotinylated spore protein. A single 40-kDa *E. cuniculi* spore protein was detected (arrow).

FIG. 1. Total spore protein from *E. cuniculi* inhibits *E. cuniculi* spore adherence to Vero cells. A spore protein lysate was generated by treating spores with SDS-boiling buffer containing reducing agent. The protein was serially diluted and added along with spores in a spore adherence assay. The data are presented as the percentages of adherence spores per field of magnification compared to control samples with lysate buffer only. Total *E. coli* protein was also used as a control. These data represent one experiment, which was repeated three times with similar results. Significance was determined using the Student *t* test.

FIG. 2. Demonstration of an *E. cuniculi* spore protein that binds Vero host cells. A total *E. cuniculi* spore protein lysate was generated, biotin labeled, TCA precipitated, and resuspended in DMEM growth medium, which was placed onto Vero host cell monolayers grown in 12-well culture plates. After incubation, the monolayers were washed of unbound protein, and a Western blot of host cell protein lysate was detected with streptavidin-conjugated alkaline phosphatase. The control lanes include biotin-labeled TCA-precipitated (TCA ppt) *E. cuniculi* spore protein and host cell protein without the addition of biotinylated spore protein. A single 40-kDa *E. cuniculi* spore protein was detected (arrow).

Global pairwise alignments of EcEnP1 and EiEnP1 show that the two proteins are 61.5% identical (Fig. 3), but database comparative analyses did not identify any other known or hypothetical protein with significant homology. In addition, both proteins have putative signal peptides, suggesting that they are transported to the cell surface; however, no transmembrane domains or lipid-binding motifs are detected in either homolog. Interestingly, previously characterized *E. intestinalis* and *E. cuniculi* spore wall proteins also lack these motifs, but they are clearly found on the surface of spores (1, 10). EcEnP1 has two predicted HBMs (HBM1, “XBBBXBXBBX” [amino acids 150 to 158] and HBM2, “XBBBXBX” [amino acids 329 to 334]), while EiEnP1 has three HBMs (“XBBBXBXBBX” [amino acids 248 to 256] and “XBBBXBX” [amino acids 193 to 198 and 322 to 327]). The “XBBBXBXBBX” motif differs slightly from the originally proposed consensus sequence; however, others previously suggested that the spacing of the basic amino acids in the motif are more important to protein-glycan interactions than the specific linear motif (16). Both EnP1 proteins are also remarkably cysteine rich (25 cysteines for EiEnP1 and 23 cysteines for EcEnP1) and may be involved in either intra- or interchain disulfide bonding. Moreover, EiEnP1 has an RGD cell attachment motif (amino acids 221 to 223), which has been shown to be a requirement for integrin association in other proteins. EcEnP1 has a conserved RGE motif in the same location (amino acids 230 to 232). At this
time, the ability of EiEnP1 or EcEnP1 to bind integrins is not known. The predicted pIs for EcEnP1 and EiEnP1 are 9.28 and 9.12, respectively.

**Western analysis of EnP1.** To further characterize EnP1, the *E. cuniculi* homolog (amino acids 20 to 356) was heterologously expressed as a histidine fusion protein in *E. coli* and purified by nickel affinity chromatography. EcEnP1-specific antibody was generated and purified from immunized rabbit antiserum by protein A/G chromatography and used in Western blotting analyses. A single 40-kDa band was detected from both *E. cuniculi* and *E. intestinalis* spore protein lysates under reducing conditions (Fig. 4A and B). The fact that the reactive band size matched the predicted molecular mass (40.5 kDa for EcEnP1 and 39.1 kDa for EiEnP1) suggests that EnP1 is not glycosylated. For confirmation, we combined *E. cuniculi* spore protein with concanavalin A (ConA) lectin conjugated to an agarose bead matrix (Fig. 4C and D). ConA has been used to detect glycosylated proteins in other studies of microsporidia (10, 26). While the control reaction showed several *E. cuniculi* proteins interacting with ConA, indicating possible posttranslational glycosylation, EcEnP1 does not react and is therefore probably not glycosylated.

**EnP1 is located in the spore wall and in the anchoring disk complex.** To determine the location of EnP1 in spores, the specific antibody was used for IEM of host cells infected with *E. cuniculi* or *E. intestinalis* spores (Fig. 5 and 6). Very little labeling occurred in the meronts that form at the edge of parasitophorous vacuoles (Fig. 5). However, in sporonts, labeling was most often noted on the developing spore wall (data not shown).

In mature spores, EnP1 is localized to two regions in both *E. intestinalis* and *E. cuniculi* spores: the spore wall and the anchoring disk complex (Fig. 5). EnP1 is expressed in both the endospore and the exposed exospore of the spore wall. In the electron-lucent endospore, EnP1 appears to be randomly dispersed and not directly associated with the plasma membrane. On the exospore, EnP1 expression appears to coincide with the development of the electron-dense layer. EnP1 localization does not appear to be specifically limited to one layer because in most mature spores, both the endospore and the exospore are equally labeled. Previous studies have shown that this protein is expressed in the endospore; however, no exposed exo-
spore labeling data were shown (17). These differences may reflect the use of different tools and techniques. Our EnP1 antibody was generated in naïve rabbits and purified from the serum prior to experimentation. Peuvel-Fanget et al. previously used serum from immunized mice for IEM (17). Nonetheless, our IEM data clearly show that EnP1 is accessible on the exposed exospore.

In addition to the spore wall, EnP1 is also found in the anchoring disk complex (Fig. 6), which consists of the polar sac and the anchoring disk. The polar sac is an electron-dense structure enclosed in a unit membrane that is closely associated with the apex of the spore. The polar filament enters the polar sac and terminates as a flared biconvex disk or funnel shape in the center of the sac. This complex is thought to play a crucial role in the rupture of the spore wall and the subsequent release of the polar tube following activation (23). In both *E. cuniculi* and *E. intestinalis*, EnP1 is localized both on the anchoring disk and in the polar sac. The labeling pattern appears to be similar between *E. cuniculi* and *E. intestinalis*. By comparison, there appears to be more labeling of the anchoring disk than the polar sac (Fig. 6).

**EnP1 inhibits spore adherence and host cell infection.** Since EnP1 contains sequence motifs that are known to bind glycans and is expressed on the surface of spores, a series of assays was conducted to determine if EnP1 is involved in spore adherence and infection. When recombinant EcEnP1 was used as an inhibitor, *E. intestinalis* spore adherence was inhibited in a dose-dependent manner (Fig. 7B). Adherence was reduced by approximately 60% with 1 μg/ml of rEcEnP1. Similar reductions were also seen using *E. cuniculi* spores (data not shown).

To determine if the HBMs of EcEnP1 are responsible for this adherence inhibition, multiple deletion mutants were constructed by site-directed mutagenesis (Fig. 7A). These include a rEcEnP1 lacking HBM1 (HBM1-DM), one lacking HBM2 (HBM2-DM), one lacking both HBMs (HBM1&2-DM), and one lacking an apparently unrelated region in the N-terminal section of the protein (N-Term-DM [“NIKKAY”] [amino acids 62 to 67]). The N-terminal deletion mutant and the HBM2 deletion mutant inhibited spore adherence by approximately 60% at 1 μg/ml. This inhibition was similar to that of the nonmutated EcEnP1 (Fig. 7B). However, the HBM1 deletion mutant and the double deletion mutant did not inhibit adher-
ence at any protein concentration assayed. These data indicate that the HBM1 motif of EcEnP1 is necessary for spore adherence to host cells. It is also possible, however, that by deleting HBM1, the structural integrity of the recombinant protein has been compromised in such as way as to limit its ability to inhibit adherence. The inability of HBM2-DM to inhibit adherence indicates that it may not contain the necessary structure for glycan binding or, perhaps, is not accessible for glycan binding.

Our current hypothesis is that spore adherence to host cells is a necessary event precedent to spore activation and host cell infection. To test this hypothesis and to determine the influence of rEcEnP1 on infection, the deletion mutant and non-mutated recombinant proteins were used in an infection assay (Fig. 7C). Following adherence of *E. intestinalis* spores in the presence of these recombinant proteins, the unbound spores were removed by washing, and incubation of host cells with attached spores was continued so that host cells became infected. The addition of EcEnP1 and N-Term-DM and HBM2-DM mutants inhibited infection by 60 to 80%. Infection was not significantly inhibited by the addition of HBM1-DM or HBM1&2-DM. These data show that exogenous EcEnP1 not only inhibits adherence but also decreases host cell infection. Moreover, the results suggest that HBM1 of EcEnP1 plays a critical role in adherence and infection.

In vitro assays were also conducted to assess the influence of anti-EcEnP1 antibodies on *E. intestinalis* adherence and infection (Fig. 8). The addition of specific anti-EnP1 antibody to the spore adherence assay decreased both spore adherence and host cell infection in a dose-dependent manner, while control antibody generated to a recombinant heat shock protein 70 (HSP70)-like protein (ECU02_0100 [amino acids 1 to 410]) (data not shown) did not affect either adherence or infection at any concentration tested. In fact, maximum inhibition of adherence (56%) and maximum inhibition of infection (46%) occurred when 1 μl/ml EnP1 antibody was used. The ability of EnP1 antibody to block spore adherence and infection in vitro reaffirms EnP1’s localization to the accessible exospore region of *E. intestinalis*. These data also suggest the potential usefulness of EnP1 as a vaccine candidate to prevent microsporidiosis.

**EnP1 attaches to host cell surfaces.** The ability of EcEnP1 to attach to in vitro-grown host cell surfaces was determined by incubating the recombinant protein with both Vero and CHO cells as well as mutant CHO cells deficient in host cell surface GAGs (6, 7). Following the incubation period, unbound protein was removed by washing. Total protein lysate was prepared from the monolayers and used in Western blotting analyses. rEcEnP1 binds to Vero cells and parent CHO cells, which have the full complement of surface GAGs (Fig. 9). In contrast, EcEnP1 failed to attach to mutant CHO cell lines pgsA-745 and pgsD-677, which are reportedly deficient in all surface GAGs and heparan sulfate, respectively. The inability of rEcEnP1 to attach to GAG-deficient cells indicates that EnP1 most likely interacts with host cell surface glycans. These data also resemble data from previous spore adherence studies where *E. intestinalis* spore attachment to these mutant CHO cells was reduced by as much as 94% compared to the non-mutated parent cells (12). Since the pgsD-677 cells are reported to be deficient in heparan sulfate, while retaining surface expression of other GAGs like chondroitin sulfate A, the inability of rEcEnP1 to attach to these cells suggests that this proposed ligand may preferentially bind heparan sulfate even though it has been shown that several exogenous sulfated GAGs are capable of preventing spore attachment (12).

**DISCUSSION**

The attachment of obligate intracellular pathogens to the host cell surface is often the first step in a series of events that leads to infection and propagation. Viral, bacterial, fungal, and parasitic pathogens employ this tactic, usually resulting in re-
Microsporidians of the genus *Encephalitozoon*, specifically, *E. cuniculi* and *E. intestinalis*, adhere to host cell surfaces in vitro prior to and during host cell infection (12, 21). The spore is activated through a still undefined mechanism. A hollow polar tube is discharged from the apical end of the spore and ultimately penetrates the host cell cytoplasmic membrane through either brute force or receptor-mediated endocytosis (8, 25). An alternate theory of infection involves the endocytosis of an attached spore into the cytoplasm followed by the rupture and release of the polar tube (4). Regardless of the mechanism, host cell infection is not known to occur without tube extrusion and the transfer of sporoplasm, which gives rise to a new generation of spores.

A recent examination of microsporidian spore adherence to host cells in vitro indicates that adherence may precede spore activation and host cell infection (12). The inhibition or alteration of the adherence process using a variety of compounds or host cell treatments indicates that *Encephalitozoon* sp. spores are utilizing host cell surface GAGs. In this study, we have identified EnP1 as being a microsporidian adherence protein. EnP1 resides on the surface of *E. intestinalis* and *E. cuniculi* spores and is a putative spore adherence ligand.

FIG. 7. *E. intestinalis* spores are inhibited from adherence to Vero host cells by recombinant EnP1. A schematic representation of the recombinant EnP1 protein and the deletion mutants, which include the nondeleted EcEnP1 protein, the HBM1 deletion mutant (HBM1-DM), the HBM2 deletion mutant (HBM2-DM), the double deletion mutant (HBM1&2-DM), and an irrelevant N-terminal deletion mutant (N-Term-DM), is shown (A). Each recombinant protein is used as an inhibitor of *E. intestinalis* spore adherence at the indicated concentration (B). Following the adherence assay, a duplicate set of coverslips was returned to culture to measure host cell infection (C). The dashed line represents the level of either spore adherence or host cell infection resulting from a control sample of 1 μg/ml nontransformed total *E. coli* protein. The data are shown as the percentage of adherence or infection compared to control samples without added inhibitors. The asterisks indicate significant differences (*P* < 0.0001). These data represent one of three experiments that were performed, with similar results.
antibody. E. cuniculi, were lysed, and Western blotting was performed using EnP1-specific antibody. Control samples received no recombinant protein (“/H11001”). After incubation, the nonbound protein was removed by washing, the monolayers were lysed, and Western blotting was performed using EnP1-specific antibody, E. c., E. cuniculi.

IEM data place EnP1 in the endospore and exospore regions of the spore wall and in the anchoring disk complex. Previous studies using antiserum derived from mice immunized with a heterologously expressed protein placed the EnP1 protein in the endospore (17). Because of the observed localization, the protein was named endospore protein 1 (EnP1). It is unclear why our localization data differ from the results of Peuvet-Fanget et al. and show protein expression in additional sites. It is possible that the different methods of antibody production may affect the results. Our antibody was purified from the serum of immunized rabbits, whereas the previous study apparently used serum from immunized mice. The fact that anti-EnP1 antibody blocks adherence, while a control antibody to the internal protein (HSP70) does not, reaffirms our IEM observation that EnP1 is externally exposed. Additional study of this unique and important protein may be necessary to reconcile the differences in expression patterns.

Given its location in the endospore and the high number of cysteine residues in EnP1, it was suggested that this protein could function in a structural capacity by using disulfide bridging to confer stability to the spore wall (17). In addition to disulfide bridging, EnP1 may directly interact with the chitinous glycans of the endospore by way of the identified HBMs. Although there is slight variability in the spacing of the basic amino acids of EcEnP1 HBM1 compared to the consensus sequence, the specific interactions of HBM motifs with various glycans may be more dependent upon the spatial arrangement of the basic amino acids than upon the interaction between specific glycans and a strict linear motif (16). This spacing variability may reflect an ability to bind a variety of glycans on cell surfaces and spore wall chitinous glycans. Clearly, additional study is required to fully characterize the ability of EnP1 to bind spore wall chitin.

The anchoring disk complex is where the polar tube attaches to the spore wall and where the spore wall ruptures, releasing the everting polar tube. One model describes the formation of a “collar” by the rotation of the anchoring disk during spore wall rupture (15). The collar then serves as a holding structure or as the conduit through which the tube everts. The mechanism that initiates rupture is unknown; however, our data show that EnP1 is expressed in high abundance on the flared biconvex anchoring disk and in the polar sac. Following the rotational mechanism of rupture, the proteins residing in this region would be transferred to the collar, where they could interact with host cell surface receptors and orient the spore such that polar tube eversion would be directed onto the host cell plasma membrane.

Early spore-staining studies used periodic acid-Schiff staining techniques of whole spores to determine that the proteins in the anchoring disk complex are highly glycosylated (23). This was confirmed using conjugated lectins, such as ConA, and electron microscopy (22). However, the data presented here indicate that even though EnP1 is expressed in the anchoring disk complex, it is likely not glycosylated, due to its inability to bind ConA. Therefore, the periodic acid-Schiff reaction of the anchoring disk complex could be due to a protein(s) other than EnP1.

Several lines of evidence suggest that EnP1 is an adhesin involved in spore adherence to host cell surfaces. First, it is accessible. If a protein is not exposed and reachable, it cannot act as a ligand. Our data clearly show that EnP1 is distributed on the exospore surface of both E. intestinalis and E. cuniculi. Second, rEcEnP1 blocks spore adherence to host cells in vitro. The ablation of adherence was dose dependent. Moreover, by removing HBM1 from rEcEnP1, all adherence inhibition was eliminated, which signifies the importance of a sequence motif known to be responsible for protein-glycan interactions. Third, rEcEnP1 attaches directly to host cell surfaces that express GAGs. These cumulative data support the idea that EnP1 is a surface adherence ligand.

Interestingly, the complete ablation of spore adherence to host cells using rEcEnP1 as an inhibitor was not achieved. One microgram of recombinant protein per milliliter yielded approximately 60% inhibition. In previous studies using exogenous GAG inhibitors, the maximal spore adherence reduction was roughly 70 to 90% of that of control samples without GAGs (12). Since complete inhibition has not been obtained, it is possible that either another spore surface ligand that acts in concert with EnP1 exists or an alternate mechanism of adherence that is independent of GAGs exists. Some viruses, for example, are known to use a combination of receptors for attachment and entry. Foot-and-mouth disease viruses use cell surface heparan sulfate to concentrate virus particles for subsequent integrin receptor binding (13).

In support of our hypothesis that adherence and infection are directly linked, the data presented herein show that when adherence is inhibited by either rEcEnP1 or specific anti-EnP1 antibody, host cell infection is reduced. If this occurs in vivo, our hypothesis will have important implications for the development of new therapeutics to treat microsporidiosis. By blocking adherence using either recombinant protein or HBM peptides, one could sufficiently reduce reinfection or, theoretically, limit dissemination. Perhaps a more effective and effi-
cient approach would be to use EnP1, or some HBM derivative, as an immunogen in a vaccine for the development of specific protective antibodies. Our data showing that rEcEnP1 rabbit antibody blocks host cell infection in vitro suggest that this approach has promise, and future studies will be directed to the development of this idea.

ACKNOWLEDGMENTS

We thank the Electron Microscopy Core Facility, James H. Quillen College of Medicine, East Tennessee State University, for consultation and technical assistance with transmission electron microscopy and Sara Davis-Hayman for critical reading of the manuscript.

REFERENCES

1. Bohne, W., D. J. Ferguson, K. Kohler, and U. Gross. 2000. Developmental expression of a tandemly repeated, glycine- and serine-rich spore wall protein in the microsporidian pathogen Encephalitozoon cuniculi. Infect. Immun. 68:2268–2275.
2. Brosson, D., L. Kuhn, G. Prensier, C. P. Vivares, and C. Texier. 2005. The putative chitin deacylase of Encephalitozoon cuniculi: a surface protein implicated in microsporidial spore-wall formation. FEMS Microbiol. Lett. 247:81–90.
3. Cardin, A. D., and H. J. Weintraub. 1989. Molecular modeling of protein-glycosaminoglycan interactions. Arteriosclerosis 9:21–32.
4. Couzinnet, S., E. Cejas, J. Schittay, P. Deplazes, R. Weber, and S. Zimmerli. 2000. Phagocytic uptake of Encephalitozoon cuniculi by nonprofessional phagocytes. Infect. Immun. 68:6939–6945.
5. Desportes, I., Y. Le Charpentier, A. Galian, F. Bernard, B. Cochand-Priollet, A. Lavergne, P. Ravisse, and R. Modigliani. 1985. Occurrence of a new microsporidian: Enterocytozoon bennetti n. g., n. sp., in the enterocytes of a human patient with AIDS. J. Protozool. 32:250–254.
6. Esko, J. D., K. S. Rostand, and J. L. Weinke. 1985. Occurrence of a new microsporidium: Enterocytozoon bennetti n. g., n. sp., in the enterocytes of a human patient with AIDS. J. Protozool. 32:250–254.
7. Esko, J. D., T. E. Stewart, and W. H. Taylor. 1985. Animal cell mutants defective in glycosaminoglycan biosynthesis. Proc. Natl. Acad. Sci. USA 82:3197–3201.
8. Foucault, C., and M. Drancourt. 2000. Actin mediates Encephalitozoon intestinalis entry into the human enterocyte-like cell line, Caco-2. Microb. Pathog. 28:51–58.
9. Giles, D. K., J. D. Whittimore, R. W. LaRue, J. E. Raulston, and P. B. Wyrick. 2006. Ultrastructural analysis of chlamydial antigen-containing vesicles evertting from the Chlamydia trachomatis inclusion. Microbes Infect. 8:1579–1591.
10. Hayman, J. R., S. F. Hayes, J. Amom, and T. E. Nash. 2001. Developmental expression of two spore wall proteins during maturation of the microsporidian Encephalitozoon intestinalis. Infect. Immun. 69:7057–7066.
11. Hayman, J. R., and T. E. Nash. 1999. Isolating expressed microsporidial genes using a cDNA subtractive hybridization approach. J. Eukaryot. Microbiol. 46:215–245.
12. Hayman, J. R., T. R. Southern, and T. E. Nash. 2005. Role of sulfated glycans in adherence of the microsporidian Encephalitozoon intestinalis to host cells in vitro. Infect. Immun. 73:841–848.
13. Jackson, T., A. M. King, D. I. Stuart, and E. Fry. 2003. Structure and receptor binding. Virus Res. 91:33–46.
14. Keohane, E. M., and L. M. Weiss. 1998. Characterization and function of the microsporidian polar tube: a review. Folia Parasitol. (Praha) 45:117–127.
15. Keohane, E. M., and L. M. Weiss. 1999. The structure, function, and composition of the microsporidian polar tube, p. 196–224. In M. Wittner and L. M. Weiss (ed.). The microsporidia and microsporidiosis. ASM Press, Washington, DC.
16. Margalit, H., N. Fischer, and S. A. Ben-Sasson. 1993. Comparative analysis of structurally defined heparin binding sequences reveals a distinct spatial distribution of basic residues. J. Biol. Chem. 268:19226–19231.
17. Peuvel-Fanget, I., V. Polonais, D. Brosson, C. Texier, L. Kuhn, P. Peyret, C. Vivares, and F. Delbac. 2006. EnP1 and EnP2, two proteins associated with the Encephalitozoon cuniculi endospore, the chitin-rich inner layer of the microsporidial spore wall. Int. J. Parasitol. 36:309–318.
18. Rostand, K. S., and J. D. Esko. 1997. Microbial adherence to and invasion through proteoglycans. Infect. Immun. 65:1–8.
19. Sandfort, J., A. Hannemann, H. Gelderblom, K. Stark, R. L. Owen, and B. Raf. 1994. Enterocytozoon bennetti infection in an immunocompetent patient who had acute diarrhea and who was not infected with the human immunodeficiency virus. Clin. Infect. Dis. 19:514–516.
20. Snowden, K. F. 2004. Zoonotic microsporidia from animals and arthropods with a discussion of human infections, p. 123–134. In D. S. Lindsay and L. M. Weiss (ed.). Opportunistic infections: toxoplasma, sarcoceysis, and microsporidia, vol. 9. Kluwer Academic Publishers, Boston, MA.
21. Southern, T. R., C. E. Jolly, and J. R. Hayman. 2006. Augmentation of microsporidia adherence and host cell infection by divalent cations. FEMS Microbiol. Lett. 260:143–149.
22. Taupin, V., E. Garenaux, M. Mazet, E. Maes, H. Denise, G. Prensier, C. P. Vivares, Y. Guerardel, and G. Metenier. 2007. Major O-glycans in the spores of two microsporidian parasites are represented by unbranched manno-oligosaccharides containing alpha-1,2 linkages. Glycobiology 17:56–67.
23. Vavra, J., and J. I. Larsson. 1999. Structure of the microsporidia, p. 7–84. In M. Wittner and L. M. Weiss (ed.). The microsporidia and microsporidiosis. ASM Press, Washington, DC.
24. Xu, Y., P. Tukvorian, A. Cali, F. Wang, H. Zhang, G. Orr, and L. M. Weiss. 2006. Identification of a new spore wall protein from Encephalitozoon cuniculi. Infect. Immun. 74:237–248.
25. Xu, Y., P. Tukvorian, A. Cali, and L. M. Weiss. 2003. Lectin binding of the major polar tube protein (PTP1) and its role in invasion. J. Eukaryot. Microbiol. 50(Suppl.):603–601.
26. Xu, Y., P. M. Tukvorian, A. Cali, G. Orr, and L. M. Weiss. 2004. Glycosylation of the major polar tube protein of Encephalitozoon hellem, a microsporidian parasite that infects humans. Infect. Immun. 72:6341–6350.