Microsporidia Spore Adherence and Host Cell Infection In Vitro

Cory A. Leonard

East Tennessee State University

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Microsporidia Spore Adherence and Host Cell Infection *In Vitro*

A dissertation

presented to

the faculty of the Department of Biomedical Sciences

East Tennessee State University

In partial fulfillment

of the requirements for the degree

Doctor of Philosophy in Biomedical Sciences

by

Cory A. Leonard

August 2013

J. Russell Hayman, Ph.D., Chair

Fred Hossler, Ph.D.

David Johnson, Ph.D.

Michael Kruppa, Ph.D.

Robert Schoborg, Ph.D.

Keywords: Microsporidia, *Encephalitozoon intestinalis*, *Encephalitozoon cuniculi*, Adherence, Infectivity, Microsporidia ADAM, MsADAM, Integrins
Microsporidia infect invertebrate and vertebrate animals. Human pathogenic microsporidia are associated with severe disease in immunocompromised individuals, and mostly asymptomatic infection in the immunocompetent. Treatment options for microsporidiosis are limited, incompletely effective, and associated with toxicity. Furthermore, microsporidia infection of healthy individuals is poorly understood, and the consequences of asymptomatic infection have not been determined. Little is known about the molecular mechanisms of microsporidia infection, but such information is essential for the development of new therapies. Spores adhere to host cell surfaces \textit{in vitro}. Our laboratory has focused on determining specific host cell and microsporidia spore surface participants in spore adherence. Our previous studies have shown that host cell sulfated glycosaminoglycans and the spore surface protein EnP1 participate in spore adherence to host cells. Additionally, \textit{in vitro} inhibition or augmentation of spore adherence decreased or increased host cell infection, respectively. These studies demonstrated the importance of spore adherence in host cell infection and began to characterize the host cell and spore determinants of adherence. The goal of this research was to further characterize host cell and spore participants in microsporidia adherence and infection of host cells \textit{in vitro}. We characterized an intracellular microsporidia protein and related antibodies for analyses of microsporidia spore surface proteins; characterized a spore surface protein, MsADAM, involved in spore adherence to and infection of host cells \textit{in vitro}; and suggested a role for host cell integrins in microsporidia adherence to and infection of host cells \textit{in vitro}. 
DEDICATION

For Doug.
ACKNOWLEDGMENTS

Thanks to the students, staff, faculty, and my committee for their many contributions to my education.

Thanks, as always, to my husband, family, and friends for their dedication, support, and encouragement.

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CHAPTER 1

INTRODUCTION

Microsporidia and Microsporidiosis

Microsporidia are eukaryotic, obligate intracellular, spore-forming, fungal-related pathogens that infect a broad range of invertebrate and vertebrate animals, as well as humans. First described as silkworm pathogens in 1857, microsporidia have long been known to cause disease in many nonhuman animals including wild and farm raised animals and domestic pets (Franzen and Müller 2001; Didier et al. 2004; Didier and Weiss 2006). The first incidence of human microsporidiosis, however, was not recorded until 1959; and only 10 human cases were reported before the onset of the human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) pandemic (Franzen and Müller 2001; Mathis et al. 2005).

Microsporidia are currently considered to be emerging, opportunistic human pathogens and are frequently associated with life-threatening diarrhea, wasting, and disseminated disease in AIDS patients (Franzen and Müller 2001; Didier 2005). Microsporidia have been found in nearly all organs of the human body and the symptoms of disseminated microsporidiosis vary by location of the infection. The clinical syndromes associated with microsporidiosis include enteropathy, keratoconjunctivitis, sinusitis, tracheobronchitis, encephalitis, interstitial nephritis, hepatitis, cholecystitis, osteomyelitis, and myositis (Kotler and Orenstein 1998).

Microsporidiosis is not restricted to HIV infected individuals, however, and HIV negative people with varying degrees of immune deficiency, including those undergoing solid organ transplant, cancer patients, those with underlying gastrointestinal disease, diabetics, young children, and the elderly may also exhibit severe or disseminated forms of the disease (Didier and Weiss 2011; Ditrich et al. 2011; Andreu-Ballester et al.
Recently, a study showed 30% prevalence (by polymerase chain reaction; PCR) of microsporidia in intestinal tissue biopsies from patients with Crohn’s disease, an inflammatory bowel disease associated with reduced peripheral blood leukocytes; while no microsporidia DNA was detected in healthy control samples (Andreu-Ballester et al. 2013). In a particularly striking example of severe and disseminated microsporidiosis in an HIV negative individual, a 57 year old man with no major immune deficiency, under medical care for diabetes and high blood pressure, suffered a brain abscess attributed to a microsporidia infection that likely spread to the brain from a primary sinus infection (Ditrich et al. 2011). Microsporidia thus has the potential to both complicate existing disease and to cause severe and life-threatening disease in humans without serious immune deficiency.

Healthy immunocompetent individuals may become infected as well, although they are typically either asymptomatic or exhibit only mild and self-limiting gastrointestinal symptoms (Van Gool et al. 1997; Didier and Weiss 2011; Sak, Brady, et al. 2011; Sak, Kváč, et al. 2011). In a study surveying 576 healthy individuals, high antibody titers against microsporidia were found in 8% of Dutch blood donors and 5% of pregnant French women (Van Gool et al. 1997). Another recent study evaluated samples from 382 healthy Czech residents and showed more directly, by microscopic stool analysis, 15% prevalence of microsporidia with no significant correlation to diarrhea (Sak, Brady, et al. 2011). In a smaller study that specifically examined samples from people with occupational exposure to animals, microscopic and PCR evaluation of urine and stool samples as well as serological analysis revealed that 14 out of 15 people were positive for microsporidia despite a lack of clinical symptoms (Sak, Kváč, et al. 2011). These studies suggest that microsporidia infection is relatively common in healthy individuals and raises the question of asymptomatic carriage in humans as a potential risk for severe disease in the event of subsequent immune suppression.
Over 1200 species of microsporidia have been identified, and 14 of these have been shown to infect humans (Didier et al. 2004). Enterocytozoon bieneusi, described in 1985, is the most common microsporidia to infect humans and the most prevalent microsporidia in HIV positive individuals (Desportes et al. 1985; Didier et al. 2004; Didier and Weiss 2011). E. bieneusi rarely causes disseminated disease and is most frequently associated with self-limiting diarrhea in immunocompetent individuals and persistent diarrhea and wasting in immunosuppressed individuals (Kotler and Orenstein 1998; Didier et al. 2004). The Encephalitozoon species are also commonly identified in humans both with and without HIV infection (Didier and Weiss 2011). Although identification at the species level was not possible at the time, an Encephalitozoon species was responsible for the first recorded case of human microsporidiosis in 1959 (Mathis et al. 2005).

Encephalitozoon intestinalis is the second most commonly reported cause of human microsporidiosis, despite its relatively recent description in 1993, and is frequently associated with HIV/AIDS (Cali et al. 1993; Didier et al. 2004; Mathis et al. 2005; Didier and Weiss 2011). Encephalitozoon cuniculi, identified in 1922, was the first microsporidia to be recognized as a parasite of mammals, and though it is less frequently identified in humans than E. intestinalis is the best-studied microsporidia and was the first mammalian microsporidia to be successfully cultured in a laboratory setting (Smith and Florence 1925; Didier et al. 2004; Mathis et al. 2005; Kotkova et al. 2013). Both E. intestinalis and E. cuniculi are associated with gastrointestinal symptoms and disseminated microsporidiosis (Didier et al. 2004; Didier and Weiss 2011). The remaining 11 species of microsporidia known to infect humans are less frequently isolated from patients and include Encephalitozoon hellem, Anncaliia algerae, A. connori, A. vesicularum, Microsporidium africanum, M. ceylonensis, Nosema ocularum, Pleistophora ronneafiei, Trachipleistophora anthropophthera, T. hominis, and Vittaforma corneae (Didier and Weiss 2011).
Treatment of Microsporidiosis

Treatment of HIV infected individuals with antiretroviral therapy (ART) has greatly reduced the incidence of opportunistic infections, including microsporidiosis. However, in areas with insufficient access to ART, diarrhea and wasting associated with microsporidiosis continue to be a concern (Mathis et al. 2005; Didier and Weiss 2011; Wumba et al. 2012). Additionally, as stated previously, not all cases of microsporidiosis are associated with HIV or any immune suppression. Treatment options for microsporidiosis are extremely limited and are not completely effective. Currently, only 2 drugs are widely used to treat microsporidiosis in humans. Albendazole, a tubulin polymerization inhibitor with parasitostatic activity, is effective against *Encephalitozoon* microsporidia but only variably effective against *E. bieneusi* (Didier et al. 2004). Fumagillin, a methionine aminopeptidase-2 (MetAP2) inhibitor that irreversibly blocks microsporidial MetAP2 and has fungicidal activity, can be used topically to treat keratoconjunctivitis due to *Encephalitozoon* species and is effective against *E. bieneusi* but causes neutropenia and thrombocytopenia in some patients (Didier et al. 2004; Anane and Attouchi 2010).

Several additional drugs are being evaluated for treatment of human microsporidiosis, including TNP-470, a semisynthetic analog of fumagillin, ovalicin, another MetAP2 inhibitor, fluoroquinolones, topoisomerase IV inhibitors, and polyamine analogs, which deregulate cell metabolism and inhibit parasite growth (Anane and Attouchi 2010). A recent study showed that mice experimentally infected with *E. cuniculi* then treated with albendazole appeared to clear the parasite, which became undetectable. Upon subsequent immune suppression, however, the mice exhibited reactivated infection, suggesting that albendazole treatment may relieve clinical symptoms but fails to eliminate microsporidial infection (Kotkova et al. 2013). Novel
more broadly effective and safe therapies are needed to combat the continuing problem of human microsporidiosis.

**Epidemiology and Transmission of Microsporidia**

The zoonotic potential of microsporidia is well documented, and contact with wild, farmed, or domestic animals may be an important risk factor for microsporidiosis (Deplazes et al. 2000; Didier et al. 2004; Mathis et al. 2005; Słodkowicz-Kowalska 2009). *E. cuniculi*, first isolated from rabbits, is a particularly important animal pathogen and frequently infects rabbits, canines, rodents, and monkeys (Smith and Florence 1925; Mathis et al. 2005; Furuya 2009). Recently, a survey of pet dogs in the United States showed an *E. cuniculi* seroprevalence rate of 22% in 125 tested serum samples (Cray and Rivas 2013).

Although *E. bieneusi* and *E. intestinalis* were originally isolated from humans, they are both also capable of infecting nonhuman mammals and *E. bieneusi* is frequently detected in birds (Desportes et al. 1985; Cali et al. 1993; Didier and Weiss 2011). Of particular interest is a 2011 study examining stool samples for *E. bieneusi* infection and genotype by PCR methods. Two hundred twenty samples taken from Chinese humans (diarrheal children), pigs, cows, and dogs revealed a 23% *E. bieneusi* prevalence in humans, and 16% prevalence in pigs, 8% in dogs, and 38% in cows (Zhang et al. 2011). Importantly, 5 of the 12 genotypes detected in this study were found in both humans and animals, strongly suggesting that microsporidia of animal origin may be infective to humans (Zhang et al. 2011).

A similar study evaluating stool samples from 100 pet dogs and 40 pet cats in Iran showed 31% prevalence for microsporidia in dogs, with 18, 8, and 5 dogs positive for *E. cuniculi*, *E. bieneusi*, and *E. intestinalis*, respectively. The cats showed 7.5% prevalence for *E. bieneusi* alone (Jamshidi et al. 2012). Cases reporting more direct evidence of zoonotic transmission of microsporidia are rare and limited in sample size.
These include a report of a 51 year old AIDS patient with chronic diarrhea and weight loss and his healthy pet cat testing positive for *E. intestinalis* by microscopy and PCR, and 1 child of 3 healthy siblings testing seropositive to *E. cuniculi* after exposure to puppies with diagnosed *E. cuniculi* infection (McInnes and Stewart 1991; Velásquez et al. 2012). Collectively, these studies provide compelling evidence that animals can harbor unapparent microsporidia infections and serve as reservoirs for zoonotic transmission.

Direct human-to-human or animal-to-human transmission of microsporidia is possible, and animal-to-animal transmission has been confirmed. However, most human microsporidia infection is thought to occur via inhalation or ingestion of spores excreted into the environment in feces or urine of infected animals (Didier et al. 2004; Mathis et al. 2005). While inhalation is probable for pulmonary microsporidiosis in the absence of gastrointestinal infection, most microsporidiosis is associated with gastrointestinal infection and it can be assumed that the majority of microsporidia transmission to humans occurs indirectly via the fecal-oral route. Microsporidia spores are resistant to temperature and humidity fluctuations and some water treatment procedures, and they are expected to persist for unknown periods of time in the environment (Didier et al. 2004; Furuya 2009). Zoonosis likely plays a role in the contamination of water, and waterfowl have been shown to shed large numbers of human pathogenic microsporidia spores in their feces (Slodkowicz-Kowalska et al. 2006; Malčeková et al. 2013).

Several studies provide evidence that human pathogenic microsporidia may contaminate water that humans routinely drink or otherwise come in contact with, including surface and ground water, water from treatment facilities, and water used for irrigation of crops (Dowd et al. 1998; Fournier et al. 2000; Thurston-Enriquez et al. 2002). Epidemiological studies support the possibility of waterborne microsporidia transmission and indicate that exposure to municipal or recreational water sources can
be a risk factor for microsporidiosis (Hutin et al. 1998; Cotte et al. 1999). Identification of microsporidial parasites in water sources contributed to their inclusion on both the National Institutes of Health Category B list of biodefense pathogens and the Environmental Protection Agency microbial contaminant candidates list of concern for waterborne transmission (Didier and Weiss 2006).

Studies indicate that microsporidiosis can also be contracted by ingestion of contaminated food (Jedrzejewski et al. 2007; Decraene et al. 2012). In 2009, an outbreak of gastrointestinal microsporidiosis affecting more than 100 people was caused by a single genotype of *E. bieneusi* and linked to the consumption of raw, prewashed cucumbers eaten by conference attendees at a hotel in Sweden (Decraene et al. 2012). Another recent study in which berries and vegetables purchased in Poland were evaluated by routine microscopic diagnostic techniques and fluorescent *in situ* hybridization (FISH) for speciation showed that *E. bieneusi*, *E. cuniculi*, and *E. intestinalis* spores can be found on fresh produce intended to be consumed raw (Jedrzejewski et al. 2007). A similar study conducted in Costa Rica also used microscopic diagnostics to demonstrate the presence of microsporidia spores on various berries and vegetables but did not identify the species found (Calvo et al. 2004). Additionally, *E. bieneusi* was detected by PCR in 8% of milk samples collected from 180 dairy cows on 45 farms in Korea; genotyping indicated that 20% of the positive samples were identical to human-infecting *E. bieneusi* strains (Lee 2008). Thus, food, as well as water, is a possible source of human infection with microsporidia, and measures for monitoring and controlling this type of contamination should be considered.
Spore Structure

The infectious form of microsporidia is the spore, the only extracellularly viable transmissible form of the organism. The spore wall of microsporidia consists of 2 distinct layers: the proteinaceous exospore, which has a generally uniform surface in human-infecting microsporidia, and the chitinous endospore, which is of uniform thickness except at the apex of the spore where the endospore wall thins considerably (Figure 1.1) (Keeling and Fast 2002). The plasma membrane lies directly adjacent to the endospore, between the spore wall and the internal contents of the spore, which include the nucleus, anterior anchoring disk, membranous polaroplast, polar filament, endoplasmic reticulum, ribosomes, relic mitochondria (mitosomes), and a large posterior vacuole (Didier and Weiss 2006). The base of the polar filament is associated with the thin-walled apex of the spore by the anchoring disk. The polar filament, polaroplast, and posterior vacuole comprise an apparatus for polar tube extrusion that is unique to the microsporidia.

Figure 1.1 Illustration of a Microsporidia Spore (adapted from Keeling and Fast 2002)
Spore Germination

When microsporidia spores are exposed to the appropriate stimuli, the polar filament is forcibly and rapidly extruded from the thin-walled anterior end of the spore in a process called germination. The spore ruptures in the vicinity of the anchoring disk and the polar filament is extruded from the spore, turning inside-out to become the hollow polar tube (Keeling 2009). The anchoring disk is the attachment point of the polar tube to the spore, and the infectious spore contents, the sporoplasm, move from inside the spore, through the polar tube, and exit the distal end of the tube as a droplet (Xu and Weiss 2005). In an event termed spore activation, various stimuli have been shown to trigger polar tube extrusion in the lab, including alterations in pH, dehydration and subsequent rehydration, hyperosmotic conditions, the presence of anions or cations, ultraviolet exposure, or peroxides (Keeling and Fast 2002; Xu and Weiss 2005).

Regardless of the stimulation that initiates spore activation, germination occurs due to a rapid influx of water into the spore and an associated swelling of the polaroplast and posterior vacuole; the pressure from this swelling moves the infectious sporoplasm into the polar tube and out of the spore, which is essentially reduced to an empty shell (Figure 1.2) (Keeling and Fast 2002; Xu and Weiss 2005). Several mechanisms have been suggested for the influx of water into spores during germination, including an increase in spore permeability to water, degradation of trehalose to glucose, and displacement of calcium within spore membranes (Pleshinger and Weidner 1985; Xu and Weiss 2005). The mechanisms for spore activation and germination in vivo are unknown, but they are likely to differ substantially from laboratory tested mechanisms, may vary significantly between microsporidia species, and may depend on host cell type. These mechanisms must occur very rapidly, however, because spore activation and polar tube eversion takes place in less than 2 seconds (Bigliardi and Sacchi 2001).
Figure 1.2 Spore Germination. (A) Spore showing the polar filament in black and the nucleus in gray, the polaroplast at the top of the spore, and the posterior vacuole at the bottom of the spore. (B) The polaroplast and posterior vacuole swell, the spore ruptures at the anchoring disk and the polar filament begins to evert. (C) Polar filament continues to evert, becoming the hollow polar tube. (D) The sporoplasm is forced into the polar tube. (E) The sporoplasm moves through the polar tube. (F) The sporoplasm emerges from the polar tube (adapted from Keeling and Fast 2002).

While it is commonly accepted that the force and speed of the everting polar tube pierces a target host cell's plasma membrane, allowing direct transfer of the infectious sporoplasm into the host cell cytoplasm, other mechanisms have been proposed for host cell infection as well. Microsporidia can be internalized into host cells by phagocytosis, and *Encephalitozoon* species were shown to be efficiently internalized by professional and nonprofessional phagocytes (Couzinet et al. 2000). It has been suggested that spores might germinate after phagocytic uptake and thus avoid phagolysosomal digestion (Franzen 2004). It was shown, however, that when phagocytic *E. cuniculi* uptake was inhibited by cytochalasin D, an actin polymerization inhibitor, the number of intracellular replicating *E. cuniculi* was identical to that found in
untreated controls, suggesting that polar tube eversion by phagocytosed spores does not significantly contribute to *E. cuniculi* infection (Orlik et al. 2010).

Alternatively, according to the “invagination model” of microsporidia host cell infection, the tip of the polar tube pushes the host cell plasma membrane into the cell interior forming an invagination; the extruded sporoplasm is injected into this invagination and part of the host cell plasma membrane pinches off around the sporoplasm to form a parasitophorous vacuole (PV) in which the microsporidia subsequently develop (Bohne et al. 2011). This model applies only to microsporidia, such as the *Encephalitozoon* species, for which intracellular development takes place inside a PV, and not to the majority of microsporidia, including *Enterocytozoon bieneusi* that develop in direct contact with the host cell cytoplasm. The invagination model of host cell infection is supported by studies that indicate that the PV in *E. cuniculi* is derived from host cell lipids and not the introduced microsporidia sporoplasm (Rönnebäumer et al. 2008). Although the specific mechanism(s) of microsporidia polar tube interaction with host cells for sporoplasm transfer remain unclear, the intracellular development of microsporidia is well described.

**Spore Development**

After the microsporidia sporoplast is introduced into the host cell cytoplasm, microsporidia development occurs in 2 main stages: merogony, a replication and division stage, and sporogony, a differentiation and maturation stage (Keeling and Fast 2002; Williams 2009). Once the sporoplast enters the host cell and begins replication by fission, the progeny are referred to as a meronts and have a large nuclear region, minimal organelles and a simple plasma membrane. An electron dense deposition of material occurs along the plasma membrane as the meronts develop into sporonts. In microsporidia that develop within PVs, meronts and sporonts can be found adjacent to the PV membrane (Bigliardi and Sacchi 2001). The final division of sporonts produces
sporoblasts, the developmental stage in which the spore wall and organelles are formed. Sporonts become mature spores, as determined by the eventual polarization of the organelles, and move into the lumen of the PV (Bigliardi and Sacchi 2001). All stages of spore development can frequently be observed within a single PV (Figure 1.3) (Mathis et al. 2005).

Figure 1.3 Electron Micrograph of The Developmental Stages of Encephalitozoon cuniculi in a Parasitophorous Vacuole in In Vitro-cultivated Human Fibroblast Cells. M, meront; P, sporont; B, sporoblast, with cross sections of the polar tube (indicated by arrow); S, mature spore (adapted from Mathis et al. 2005).
Infected cells typically rupture, releasing mature spores, a process that may be facilitated by specific microsporidia proteins, such as the putative divergent hemolysin homologue encoded by *E. cuniculi* (Williams 2009). Interestingly, a recently discovered nematode pathogenic microsporidia that replicates in direct contact with host cell cytoplasm appears to be able to “leak” spores from infected epithelial cells by disrupting the host cell cytoplasm without causing cells lysis, demonstrating that nonlytic infections may also occur in other non-PV forming microsporidia (Estes et al. 2011). The newly released mature spores may infect neighboring cells, disseminate within the host, or be excreted into the environment.

**Specific Aims**

Microsporidia spores adhere to host cell surfaces *in vitro*. The molecular events and processes that determine or result from this adherence are poorly understood. Previous studies showed the adherence process was mediated in part by host cell surface sulfated glycosaminoglycans (GAGs) and the microsporidia spore wall protein EnP1 (Hayman et al. 2005; Southern et al. 2007). Importantly, when microsporidia adherence to host cells was inhibited by adding exogenous sulfated GAGs or recombinant EnP1 to adherence assays, host cell infection was also reduced (Hayman et al. 2005; Southern et al. 2007). In another previous study, the addition of magnesium or manganese divalent cations to adherence assays increased spore adherence, resulting in a correlating increased host cell infection (Southern, Jolly, and Hayman 2006). These data shape our hypothesis that spore adherence is an integral event in microsporidia infection of host cells *in vitro*. Complete ablation of spore adherence and infection was not achieved using exogenous sulfated GAGs or recombinant EnP1, however, indicating that additional spore and/or host cell determinants of adherence and infection may exist.
The goal of this research is to identify host cell and spore participants in microsporidia adherence and infection of host cells *in vitro*. The specific aims of these studies are to i) characterize an intracellular microsporidia protein and related antibodies to use in comparative analyses of microsporidia spore surface proteins, ii) identify putative microsporidia spore surface proteins that participate in spore adherence to and infection of host cells *in vitro*, and iii) examine the role of host cell integrins in microsporidia adherence to and infection of host cells *in vitro*. 
CHAPTER 2

EXPRESSION AND LOCALIZATION OF AN HSP70 PROTEIN IN THE MICROSPORIDIAN ENCEPHALITOZOOON CUNICULI

Carrie E. Jolly, Cory A. Leonard, and J. Russell Hayman*

Department of Microbiology, East Tennessee State University, James H. Quillen
College of Medicine, Johnson City, TN 37614

*Corresponding author: Mailing address: ETSU/JHQ-COM, Department of Microbiology, Box 70577, Johnson City, TN 37614. Phone: (423) 439-6313. Fax: (423) 439-8044. Email: hayman@etsu.edu

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Abstract

Microsporidia spore surface proteins are an important, under investigated aspect of spore/host cell attachment and infection. For comparison analysis of surface proteins, we required antibody specific for an intracellular protein. An endoplasmic reticulum associated heat shock protein 70 family member (Hsp70; ECU02_0100; ‘C1’) was chosen for further analysis. DNA encoding the C1 hsp70 was amplified, cloned and used to heterologously express the C1 Hsp70 protein, and specific antiserum was generated. Two-dimensional Western blotting analysis showed that the purified antibodies were monospecific. Immuno-electron microscopy of developing and mature *E. cuniculi* spores revealed that the protein localized to internal structures and not to the spore surface. In spore adherence inhibition assays, the anti-C1 antibodies did not inhibit spore adherence to host cell surfaces, whereas antibodies to a known surface adhesin (EnP1) did. In future studies, the antibodies to the ‘C1’ Hsp70 will be used to delineate spore surface protein expression.
Introduction

Microsporidia are spore-forming, obligate intracellular divergent fungi with an extensive host range that includes most vertebrates and invertebrates. Although the first species of microsporidia was described over 150 years ago, microsporidiosis was rarely diagnosed in humans prior to the AIDS pandemic. Today, microsporidia are recognized as opportunistic pathogens of humans [1]. Most microsporidia infections in humans are thought to arise via the fecal-oral route. Ingestion of the environmentally stable spores leads to primary infection in the small intestine where replication of the organisms results in destruction of the epithelium. Therefore, the most common clinical manifestations of microsporidiosis are self-limiting diarrhea in immunocompetent individuals and persistent diarrhea perhaps leading to a wasting syndrome in the immunocompromised [2].

All microsporidia possess a unique invasion apparatus known as the polar tube or polar filament, which must be discharged in order to infect the host cell. Upon extrusion, the polar tube penetrates the host cell plasma membrane and allows the passage of infectious sporoplasm from the spore through the hollow polar tube into the host cell cytoplasm where replication occurs. We hypothesize that infection of the host cell is facilitated by adherence of the microsporidia spore to the host cell surface prior to or during the activation process. Our previous studies have demonstrated that microsporidia spores of the genus *Encephalitozoon* adhere to the host cell surface in vitro through at least one mechanism involving host cell glycosaminoglycans [3]. In vitro spore adherence and host cell infectivity assays demonstrate that addition of exogenous sulfated glycosaminoglycans to the culture medium results in decreased spore adherence and decreased number of infected host cells. Our studies indicate a direct association between microsporidia adherence to the host cell surface and infectivity.
To understand the mechanism of microsporidia adherence, we have turned our attention to identifying possible ligands on the spore surface. We are searching the *Encephalitozoon cuniculi* genome database for genes that encode proteins with recognizable adhesion domains. Identified proteins are recombinantly expressed, purified, and used for antibody production. We are using previously developed assays to evaluate the recombinant proteins and their corresponding antibodies as potential inhibitors of spore adherence and/or host cell infectivity. For comparison purposes, we require a microsporidia protein that is not located on the spore surface and does not inhibit spore adherence or infectivity. Because heat shock proteins (Hsps) are typically found in the cytosol, ER, and mitochondria of a cell [4], we chose to examine the Hsp70-related proteins from *E. cuniculi* as potential candidates. In this study, we demonstrate by transmission immunoelectron microscopy that the Hsp70-related protein (ECU02_0100) is located in internal structures of the spore. We also show that antibodies against the recombinant Hsp70-related protein C1 do not significantly inhibit spore adherence or host cell infection *in vitro*. Recombinant Hsp70 protein and the antibodies against this protein will be used for comparative purposes in our quest to identify possible microsporidia spore ligands that function during adherence.

**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 microsporidia 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 µm/ml), and 2% fetal bovine serum.
(BioWhittaker) in 5% CO$_2$ at 37°C. Microsporidia spore propagation and purification were performed as previously described [5].

**Recombinant Protein Expression and Antiserum Production**

The gene encoding the “C1” Hsp70-related protein (ECU02_0100) was PCR amplified from *E. cuniculi* genomic DNA using the following primers; 5’-GGAATTCATGAACAAGGTATGCTAG-3’ and 5’-ACTCGAGGAGTTCTCTCCCTATTTCC-3’. The amplicon was cloned into the pET21a vector (EMD Biosciences, Inc., Madison, WI) using restriction endonucleases and ligation. Following transformation into *Escherichia coli* Rosetta Gami cells (EMD Biosciences) and induction with IPTG (isopropyl-$\beta$-D-thiogalactopyranoside), the bacterial harvest was sonicated in PBS containing 5% SDS and 2% $\beta$-mercaptoethanol. SDS-PAGE gels were Coomassie stained, and Western analysis was performed using a histidine-tag-specific antibody (Sigma-Aldrich, St. Louis, MO) to confirm recombinant protein expression.

For purification, the bacterial pellet was sonicated in column chromatography binding buffer containing 8 M urea and 20 mM imidazole in 1X phosphate buffer saline. The supernatant was applied to an equilibrated nickel affinity column (GE Biosciences, Piscataway, NJ). Following washes with binding buffer, the recombinant protein was eluted with 1X phosphate buffer saline containing 8 M urea and 300 mM imidazole. Fractions containing recombinant protein were combined and dialyzed against 10 mM Tris buffer (pH 7.4) with 0.5 mM EDTA. The resulting dialysate was centrifuged, and the supernatant containing the protein was pooled and stored at -20°C for further use.

For antiserum production, naïve rabbits were immunized with the recombinant C1 Hsp70-related protein using a 56-day immunization protocol conducted at a
commercial facility (Proteintech Group, Inc., Chicago, IL). Antibodies from both pre-
and post-immunized rabbits were purified from serum using protein A/G affinity
chromatography according to the manufacturer's recommendations (Thermo Fisher
Scientific, Rockford, IL).

**Spore Adherence Assays**

Microsporidia spore adherence assays were performed as previously described
[3]. RK13 cells were seeded onto round glass coverslips in 12-well plates and grown to
confluence. To test adherence, purified diluted anti-C1 antibodies (1 mg/ml) or anti-
EnP1 antibodies (1 mg/ml) [6] were incubated with *E. cuniculi* spores in medium on
RK13 host cells for 4-hours on ice. The unbound spores were removed by washing with
PBS, and the bound spores were quantified by immunofluorescence as described [6].
The results are expressed as the percentage of adherent spores relative to control
samples. Statistical significance was determined using the Student's *t* test.

**SDS-PAGE Analysis and Western Blotting**

One-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (1D-
SDS-PAGE) was performed with purified spore protein as previously described [6]. For
two-dimensional (2D) SDS-PAGE analysis, 1 x 10^8 purified *E. cuniculi* spores were used
for protein sample preparation following the procedures and buffers recommended in
the ReadyPrep 2D-Starter Kit (Biorad; Hercules, CA) with slight modifications. The
spore pellet was digested for 30 minutes at boiling temperature in denaturing buffer
containing 0.05% SDS and 0.1% 2-mercaptoethanol. The supernatant was transferred
to a new tube, and the free SDS was removed using the SDS-Out Reagent
(Pierce/Thermo Scientific; Rockford, IL). The buffer was exchanged for the
ReadyPrep2D Rehydration/Sample buffer using a Microcon (YM-10) Centrifugal Filter
Device (Millipore; Billerica, MA). The buffer volume equivalent of 2.5 x 10^8 spores was
used to rehydrate two 11 cm pH 4-7 IPG strips according to the ReadyStrip IPG protocol (Biorad). The strips were focused using a Biorad Protean IEF cell and the standard recommendations for programming. For the second dimension, the gel strips were equilibrated in the kit Equilibration Buffer I and II and pre-cast Criterion 8-16% Tris-HCl SDS-PAGE gels (Biorad) were used. For Western blots, the gels were transferred as described [6].

To identify the Hsp70-related proteins, gel spots from Coomassie stained 2D-SDS-PAGE gels were excised and submitted for commercial protein identification by trypsin digestion and tandem mass spectrometry using nano-liquid chromatography/tandem mass spectrometry (Midwest Bio Services, Overland Park, KS). The acquired data were analyzed using Sequest database searching software.

Results

Cloning and Heterologous Expression of *E. cuniculi* Recombinant Hsp70-related Protein

To identify an internal protein for comparison to spore surface proteins, we focused on heat-shock proteins (Hsp), which are usually located internally in the cytosol, endoplasmic reticulum, or mitochondria. Analysis of the *E. cuniculi* genome database revealed several candidate Hsp70-related genes, some of which have been characterized including the canonical mitochondrial organelar heat shock protein [7-9]. However, for our purposes, we selected a highly expressed Hsp70 family member from *E. cuniculi* (ECU02_0100; ‘C1’), which contains all three Hsp70 protein family signature motifs of conserved family members identified by Prosite database scanning [10]. In addition, this protein contains an N-terminal signal peptide allowing it to be translocated into the endoplasmic reticulum (ER) and a C-terminal ER targeting sequence (“REEL”), which would retain the protein in the ER.
The open reading frame that encodes from the N-terminal methionine residue to the C-terminal leucine residue immediately prior to the termination codon of C1 was amplified, cloned, and expressed in *E. coli*. The expressed recombinant protein was similar to the predicted size of 76.2 kDa (Figure 2.1A) and was used to immunize naïve rabbits. In one-dimensional Western blot analysis using purified spore protein from both *E. intestinalis* and *E. cuniculi*, the anti-C1 Hsp70 protein antibodies recognized a single protein band from both species (Figure 2.1B). However, our analysis of the *E. cuniculi* genome database identified a second Hsp70-related protein with a predicted size of 74.8 kDa (ECU03_0520; 'B1'). Because the masses of these two proteins are so similar and the predicted amino acid identity is 28.3%, the single band observed in 1D Westerns may represent recognition of both the C1 and B1 Hsps. Such reactivity would be difficult to distinguish using one-dimensional SDS-PAGE. Therefore, two-dimensional SDS-PAGE electrophoresis and Western blotting analysis were performed with purified *E. cuniculi* spore proteins and the anti-C1 Hsp70 antibodies. Matrix-assisted laser desorption ionization mass spectrometry analysis of trypsin digested Coomassie gel spots identified both the C1 and B1 Hsp70 proteins (Figure 2.1C). Peptide coverage represented 38 and 24% of the C1 and B1 proteins, respectively. Interestingly, the B1 protein, which is predicted to be 74.8 kDa, is slightly larger in mass on the 2D SDS-PAGE gel than the predicted 76.2 kDa C1 protein. It is possible that the removal of the signal peptide from the C1 could account for the size discrepancy. Nonetheless, Western blotting of the two dimensional SDS-PAGE shows that the anti-C1 Hsp70 antibodies are specific for the C1 Hsp70-related protein and do not cross react with the similar sized B1 protein (Figure 2.1D).
Figure 2.1 Heterologous Expression of *E. cuniculi* C1 Hsp70 Protein as a Histidine Fusion Protein in *E. coli* and Western Blot Analysis Using Purified C1 Antibodies. A Coomassie stain SDS-PAGE gel (A, right panel) of uninduced and IPTG induced expression of the recombinant protein shows a ~76 kDa protein in the induced lane. A Western blot of the SDS-PAGE gel was performed using histidine-tag-specific antibodies (A, left panel) confirmed the recombinant protein induction. (B) A single ~76 kDa band was detected on 1D Western blot of *E. cuniculi* and *E. intestinalis* total spore protein using a purified C1 Hsp70 protein antibodies. (C) The C1 (ECU02_0100) and B1 (ECU03_0520) Hsp70-related proteins were identified from a Coomassie stained 2D SDS-PAGE gel of *E. cuniculi* total spore protein by MALDI-MS analysis of trypsin digested gel spots. (D) Western analysis of the 2D gel using the C1 specific antibodies detected only the C1 protein (D).
Immuno-localization of C1 Hsp70-like Protein in *E. cuniculi* Spores

To confirm that the C1 Hsp70-like protein does not localize to the spore wall, immuno-gold labeled transmission electron microscopy (immuno-TEM) of *E. cuniculi* infected RK13 cells was performed using the purified anti-C1 Hsp70 antibodies. The antibodies recognize protein in both immature (Figure 2.2A), and mature spores (Figure 2.2B). The C1 Hsp70 protein does not appear to be located in one specific area, but rather is spread throughout the cytoplasm. In microsporidia, the membranous ER and associated ribosomes of spores is difficult to visualize, especially in immuno-localization TEM resin. However, in structural studies, the ER has been located both surrounding the nucleus and in other parts of the cytoplasm [11]. The immuno-labeling of the C1 Hsp70 protein in Figure 2 appears to match the ER structural profile. No significant immuno-labeling was evident on the outside surfaces of developing or mature spores or within host cells that did not contain parasitophorous vacuoles (data not shown).

![Figure 2.2 Immuno-TEM of *E. cuniculi* Infected Rabbit Kidney Cells.](image)

The infected cells were prepared according to the protocol described. Ultrathin Lowicryl resin sections were cut and reacted with C1 Hsp70 specific antibodies (1:10) and a secondary antibody conjugated to 15-nm gold particles (1:200). The developmental stages include immature meronts and sporonts (the lower and upper spores in “A”, respectively) and mature spore (B). Abbreviations are as follows: PTC, polar tube coil; N, nucleus; PV, posterior vacuole; AD, anchoring disk. Each bar indicates a 500-nm scale.
Anti-C1 Antibodies do Not Inhibit *E. cuniculi* Spore Adherence to Host Cells

Previous studies have shown that microsporidia infection of host cells may involve an initial attachment of spores to host cell surfaces, which precedes spore activation, germination, and polar filament extrusion [3, 6]. Spore attachment, and thus host cell infection, can be inhibited with the addition of either sulfated glycosaminoglycans, a common host cell surface glycan, or exogenous endospore protein-1 (EnP1). EnP1 is found in both the endospore and exospore regions of spore walls and is an adherence ligand potentially involved in the spore activation process [6, 12]. Addition of either recombinant EnP1 or anti-EnP1 antibodies to spore adherence assays significantly reduces the number of adherent spores. To confirm that the anti-C1 Hsp70 antibodies, which specifically recognize the non-surface associated ER Hsp70 protein, do not affect spore attachment, spore/host cell adherence assays were performed using both the anti-C1 antibodies and the anti-EnP1 antibodies (Figure 2.3). Adding increasing amounts of anti-EnP1 antibodies leads to dose-dependent decreases in spore adherence, as was expected. However, equivalent quantities of anti-C1 antibodies have no effect on spore adherence to host cell surfaces, confirming that the internally located C1 Hsp70 is not involved in adherence.

Discussion

Previous studies involving Hsp70 proteins of microsporidia have revolved around phylogeny, in part due to the fact that microsporidia are “amitochondriate” organisms [7-9, 13]. During microsporidia genome sequencing efforts, a mitochondria-associated heat shock protein was identified, despite the fact that microsporidia have no identifiable mitochondria organelle. Studies involving this Hsp eventually identified an “ancient” mitochondria organelle called a mitosome [9, 14]. This discovery placed microsporidia in a small group of amitochondriates, which includes *Giardia intestinalis*, *Trichomonas vaginalis*, and *Entamoeba histolytica* [14, 15]. Further studies revealed that several
iron-sulphur cluster assembly proteins were located in the mitosome based on their co-localization with the mitosome-associated Hsp70 family member [16].

![Graph showing spore adherence control percentage against antibody dilution.](image)

**Figure 2.3** Purified Antibodies Specific for the Internal C1 Hsp70 Protein do Not Inhibit *E. cuniculi* Spore Adherence to Rabbit Kidney Host Cells. Increasing concentrations of C1 antibodies (open circles) or EnP1 specific antibodies (open triangle) were incubated with spores in the presence of confluent host cells. Control samples excluded antibodies. After 4-hours, unbound spores were removed by washing and the bound spores were quantified by immunofluorescence assay. Each antibody dilution sample was performed in triplicate. Statistical significance is indicated with asterisks (*P* < 0.0001).

Most cells have multiple members of the Hsp70 family of heat shock proteins that function in a variety of manners. The most recognized functions of Hsp70s are to assist in protein folding and trafficking and coping with protein denaturation in response to increased stress, such as heat [4]. Interestingly, studies have shown that within the cell, Hsp70 family members may also exert a pro-survival affect, combating the intrinsic apoptotic pathway activation in neuronal cells [17]. Hsp72 inhibits the release of
cytochrome-c from the mitochondria, inhibits apoptosis inducing factor translocation to the nucleus, and interferes with recruitment of procaspase-9 into the apoptosome [18]. Recently, it has also been shown that some Hsp70 family members can be released from cells to serve as a physiological alarm signal for cell trauma or to act as pro- or anti-inflammatory mediators [19-21]. Either through active transport or passive release due to cellular destruction, the released Hsp proteins exert their affect beyond the cell from which they originated. In our immuno-TEM analysis, we did not see significant labeling in any host cell organelle. Nor, was there significant labeling in the extracellular spaces within the parasitophorous vacuole indicating that the ER associated Hsp70-like protein may not be secreted. It remains to be seen whether the *E. cuniculi* cytosolic Hsp70-related protein (ECU03_0520; B1), which does not contain an ER retention signal or a signal peptide, is released from developing or mature spores.

The C1 Hsp70 protein contains an ER retention signal at the C-terminal end. Although the ER has not been extensively studied in microsporidia, structural studies have identified the ER as membranous elements arranged in parallel cisternae covered by ribosomes [11]. These elements both surround the nucleus and are dispersed throughout the cytoplasm. Both the spore-wall and polar-tube proteins have predicted signal peptides for ER translocation and trafficking [22-24]. Recent studies indicate that microsporidia have the necessary machinery, albeit reduced and modified, for translocation of polypeptide chains into the ER [25]. Furthermore, Golgi complex studies in microsporidia suggest they do not create budding vesicles with typical eukaryotic coat proteins, but have instead tubular networks that directly connect to the ER, the polar tube membrane, and the plasma membrane [26]. Thus, the extensive network of membranes would be distributed throughout the spore cytoplasm. The varied immuno-EM cytosolic labeling of C1 Hsp70 protein shown here would be indicative of an extensive membranous ER/Golgi secretory pathway.
In summary, we have cloned and expressed an ER associated member of the Hsp70 family from *E. cuniculi*. Antibodies generated against this protein specifically recognize C1 Hsp70 from microsporidia and localize it to internal structures of spores. Moreover, the anti-C1 antibodies did not inhibit spore adherence. As a result, the C1 Hsp70 protein and the antibody are important tools for research in the field of microsporidia and will be used as internal markers for comparisons during our studies on spore adherence.

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References


CHAPTER 3

MICROSPORIDIA ADAM FAMILY MEMBER (MICROSPORIDIA ADAM, MSADAM) IS INVOLVED IN *ENCEPHALITOZOOON INTESTINALIS* ADHERENCE AND HOST CELL INFECTION

Cory A. Leonard#, Carrie E. Jolly#, and J. Russell Hayman

Department of Biomedical Sciences, East Tennessee State University, James H. Quillen College of Medicine, Johnson City, TN 37614

*Corresponding author: Mailing address: ETSU/JHQ-COM, Department of Biomedical Sciences, Box 70577, Johnson City, TN 37614. Phone: (423) 439-6313. Fax: (423) 439-8044. Email: hayman@etsu.edu

# Authors contributed equally.

Key Words: ADAM, MsADAM, microscopy, spore, microsporidia, *Encephalitozoon intestinalis"
Abstract

Microsporidia spore adherence is a mechanism that modulates host cell infection in vitro. To better understand adherence, we focused on determining which microsporidia proteins are involved in this process. Examination of the *Encephalitozoon cuniculi* genome database revealed a gene that encodes a protein with sequence homology to members of the ADAM (a disintegrin and metalloprotease) family of type I transmembrane glycoproteins. ADAM proteins are involved in a variety of biological processes including cell adhesion and proteolysis. Like other ADAM family members, the microsporidia ADAM protein (MsADAM) has a putative signal peptide, a pro-domain, a metalloprotease domain, a disintegrin domain, and a transmembrane domain. Immunoelectron microscopy of host cells infected *in vitro* with *E. cuniculi* and *E. intestinalis* demonstrates that MsADAM localizes to the plasma membrane, exospore and polar sac-anchoring disk regions of *E. intestinalis* spores and to the polar sac-anchoring disk complex of *E. cuniculi* spores. Addition of exogenous MsADAM reduces adherence and infection of *E. intestinalis*, but not *E. cuniculi*. These data suggest a species-specific role for MsADAM in the adherence and infection process.
**Introduction**

Microsporidia are spore-forming, obligate intracellular, fungal-related parasites that have a broad host range encompassing most invertebrates and vertebrates, including humans. Environmentally, microsporidia are typically found in water sources and owe their survival to the rigid external spore wall, which consists of three layers: the external proteinaceous exospore, the protective chitinous endospore, and an inner plasma membrane. Ingestion of spores may lead to primary infection of the gastrointestinal tract, resulting in intestinal cell sloughing and persistent diarrhea in immunocompromised humans, particularly those infected with human immunodeficiency virus (HIV) [1]. Some microsporidia species, including the *Encephalitozoon* species, can disseminate to other organs from the gastrointestinal system [2]. Furthermore, microsporidia infections have been documented in immunocompetent humans, including travelers and contact lens users [3,4].

Microsporidia infect cells via a unique process involving extrusion of a hollow polar filament (or tube) from a coiled position within the spore. In response to an undefined stimulus in the gastrointestinal tract, the spore forcibly extrudes the polar tube, which pierces, or is taken in through, the host cell membrane. The contents of the spore are then transferred via the polar tube into the host cell cytoplasm. Following infection of the host cell, spore propagation and development result in host cell rupture and release of mature spores into the intestinal lumen. These mature spores may infect nearby cells, disseminate to other organ systems, or be excreted from the body into the environment.

Spores adhere to host cell surfaces *in vitro*. In previous studies, we have shown that the adherence process is mediated in part by host cell surface sulfated glycosaminoglycans (GAGs) [5]. Spore adherence was inhibited by the addition of exogenous GAGs to cell culture media in *in vitro* assays for microsporidia host cell adherence and was also significantly reduced in GAG deficient host cells, compared to
host cells with normal GAG expression. Importantly, when adherence was inhibited, host cell infection was also reduced. In other studies, we have shown that the addition of magnesium or manganese divalent cations during application of microsporidia to host cells augments spore adherence, resulting in a correlating increased host cell infection [6]. These data shape our hypothesis that spore adherence is an integral event in the infection of host cells by microsporidia.

To better understand the mechanism of adherence, we have focused on determining which host and microsporidia proteins are involved in adherence. Previously we have shown that a microsporidia spore surface protein EnP1 participates in microsporidia adherence and infection of host cells [7,8]. However, complete ablation of spore adherence to host cells was not achieved using exogenous EnP1 as an inhibitor of adherence and infection, suggesting that additional or alternative spore determinants of host cell adherence and infection may exist. Examination of the Encephalitozoon cuniculi genome database for putative adherence proteins revealed a gene encoding a protein with sequence homology to members of the ADAM (a disintegrin and metalloprotease) family. ADAMs are type I transmembrane proteins with multiple domains responsible for several physiological functions, including proteolysis, adhesion, fusion, and signaling. These domains include a pro-domain, a metalloprotease domain, a disintegrin-like domain, a cysteine-rich domain, an EGF-like domain, a transmembrane domain, and cytoplasmic domains (Figure 3.1).

Approximately half of the 40 known ADAM family members contain an apparently functional catalytic consensus sequence (HEXXHXXGXXH) in their metalloprotease domains. Active metalloprotease domains are responsible for the cleavage of cell surface molecules on the same cell [9] or neighboring cells [10]. One of the most studied ADAM cleavage targets is tumor necrosis factor-α, which is released from the cell surface by the tumor-necrosis-factor-converting enzyme (TACE; ADAM 17) [11,12].
The disintegrin domains of ADAMs are homologous to snake venom disintegrins and associate with integrins. ADAM 15, for example, can bind several integrins in both an RGD dependent (αvβ3) [13] and independent (α9β1) [14] manner. In addition, ADAMs 2 and 3 may participate in sperm-egg binding by interacting with integrin molecules on the surface of eggs [15]. This interaction may involve a specific region of the disintegrin domain called the integrin binding loop [16]. However, this loop is not the sole region responsible for target specificity, and the cysteine-rich domain is also important in cell adhesion [17,18].

Functional assays suggest the presence of proteases in microsporidia. Examination of soluble extracts from immature spores revealed four prominent protein bands on zymograms, two of which were identified as aminopeptidases [19]. Based on azocasein assays including various ions, these studies also predicted the presence of metallopeptidases in microsporidia [19]. The possible presence of protease activity or integrin binding activity in the microsporidia ADAM family protein suggests that this type of protein might play a role in host cell adherence and infection. In this study, we characterize a novel microsporidia ADAM family member (microsporidia ADAM, MsADAM) by examining the sequence characteristics and localization of MsADAM in *E. intestinalis* and *E. cuniculi* spores. Our results show that in *E. intestinalis* spores, MsADAM is located both at the spore surface and in other structures inside the spore. In *E. cuniculi* spores, however, MsADAM is absent from the spore surface and is expressed only in internal spore structures. Importantly, the addition of exogenous recombinant MsADAM to assays of spore adherence reduced adherence of *E. intestinalis* spores, but not *E. cuniculi* spores, to host cells. The MsADAM-dependent inhibition of adherence of *E. intestinalis* spores resulted in a reduction of host cell infection, suggesting a species-specific role for MsADAM in the microsporidia adherence and infection process.
Materials and Methods

Microsporidia Propagation and Purification

*E. cuniculi* and *E. intestinalis* were propagated in rabbit kidney cells (RK-13; ATCC CCL-37) or African green monkey kidney cells (Vero; ATCC CCL-81) as previously described [20]. Briefly, subconfluent RK-13 or Vero monolayers grown in 75 cm² tissue culture flasks were incubated with either *E. cuniculi* (RK-13 cells) or *E. intestinalis* spores (Vero cells) for 10-12 days with growth medium replacement as needed. Cultures were maintained in 5% CO₂ at 37°C in growth medium consisting of Dulbecco's modified Eagle's medium (BioWhittaker; Walkersville, MD) supplemented with L-glutamine (2 mM), penicillin (100 U ml⁻¹), streptomycin (100 U ml⁻¹), Amphotericin B (0.25 mcg ml⁻¹), and 2% fetal bovine serum. After collection of infected host cell debris in growth medium, spores were harvested by centrifugation at 3500 RPM for 10 minutes and purified from the host cell debris by washing with 0.25% sodium dodecyl sulfate (SDS) followed by several washes in sterile water. Spores were counted and stored in sterile water at 4°C.

MsADAM Sequence Comparisons to ADAM Family Members

Sequence identities were determined by global pairwise alignment of both whole protein sequences from ADAM family members and each individual domain sequence independently using the LALIGN program [21].

Cloning and Recombinant Protein Expression

DNA encoding the region from the pro-domain to the transmembrane-domain of MsADAM (amino acids 21-527), the MsADAM pro-domain (amino acids 21-137), and the MsADAM metalloprotease domain (amino acids 140-362) was amplified by PCR using genomic DNA from *E. cuniculi* infected RK-13 cells and the following oligonucleotide primers: MsADAM pro-domain to transmembrane domain (5'
GGAATTCTTCAGGCCGATGGTAAG-3' and 5'-
ACTCGAGTGTAGCCTGCTGAATTGCTGTCTC-3'), MsADAM pro-domain (5'-
GGAATTCTTCAGGCCGATGGTAAG-3' and “5'-
GTACTCGAGGACGTTTCCCCTCCTCATTCTC-3’), and MsADAM metalloprotease domain (5’-GGAATTCCAAAGAGTCATCAAAGTG-3' and 5’-
GTACTCGAGTATCTCGCCTATTTGGATT-3’).

The resulting PCR products were individually cloned into the pET-21a vector (Novagen; San Diego, CA), which places a histidine tag on the terminal end of the recombinant proteins. *Escherichia coli* XL1-Blue (Stratagene; La Jolla, CA) was used as the host strain for plasmid propagation. After verifying the DNA sequence, the plasmid was transformed into *E. coli* Rosetta-gami cells for protein expression (EMD Chemicals, Inc.; San Diego, CA). Recombinant protein expression was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to the culture for 4 hours at 37°C with shaking. Following induction, bacteria were harvested, resuspended in binding buffer (1X phosphate buffer pH 7.4, 20 mM Imidazole, and 8 M urea) and lysed by sonication. Protein lysates from induced and non-induced bacteria cultures were separated by sodium lauryl dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and compared via Coomassie Brilliant Blue R staining (Sigma-Aldrich; St. Louis, MO) or transferred to nitrocellulose membrane for Western analyses of expression of the histidine-tagged recombinant MsADAM protein (rMsADAM; amino acids 21-527), MsADAM pro-domain (rPRO; amino acids 21-137) and MsADAM metalloprotease domain (rMET; amino acids 140-362). Western analyses were conducted using a 1:500 dilution of mouse monoclonal anti-polyhistidine antibody (Sigma-Aldrich; St. Louis, MO), and a 1:2000 dilution of rabbit anti-mouse alkaline phosphatase-conjugated antibody (Southern Biotech; Birmingham, AL). BCIP (5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt) and NBT (nitro-blue tetrazolium chloride) were used for detection.
Recombinant Protein Purification and Antibody Production

Nickel affinity chromatography purification of histidine-tagged rMsADAM, rPRO, and rMET from *E. coli* was performed according to the manufacturer’s protocol using HiTrap HP (GE Healthcare; Piscataway, NJ) chromatography. Elution fractions of each recombinant protein were pooled, dialyzed overnight against 100 mM NaCl, 10 mM Tris, pH 8, and concentrated with a Centriplus centrifugal filter (Millipore; Billerica, MA). Protein concentrations were determined using a BCA Protein Assay Kit per manufacturer’s instructions (Pierce; Rockford, IL). Nickel affinity purified rMsADAM was provided to Proteintech Group Incorporated (Chicago, IL) for polyclonal antibody production in two rabbits. Total IgG antibodies were purified from the rabbit pre-immunization bleed and final bleed sera according to the manufacturer’s protocol for Immobilized Protein A/G Agarose (Pierce; Rockford, IL). The animal protocol for antibody production was reviewed and approved by the University Committee on Animal Care at the James H. Quillen College of Medicine, East Tennessee State University.

Western Analyses of Spore Protein

*E. cuniculi* and *E. intestinalis* spore proteins were obtained by boiling the spores for 15 minutes in lysis buffer (5 % SDS, 60 mM Tris-HCl pH 6.8, 10 % glycerol, and fresh 2-mercaptoethanol added to a final concentration of 15 % (v/v)). Following centrifugation, the resulting total protein lysate was separated by SDS-PAGE. Western analyses were conducted using a 1:500 dilution of protein A/G purified polyclonal rabbit antibodies against rMsADAM and a 1:2000 dilution of goat anti-rabbit alkaline phosphatase-conjugated antibody (Southern Biotech; Birmingham, AL).

Identification of the ~143 kDa *E. cuniculi* Spore Protein

A sheet of nitrocellulose membrane was incubated for one hour with a 1:100 dilution of anti-rMsADAM antibodies prepared in Tris buffered saline (TBS), pH 7.4, plus
0.05% Tween-20 (1X TBST). After blocking the membrane with 5% non-fat dry milk in 1X TBS, the membrane was incubated overnight in a solution of protein extracted from lysed *E. cuniculi* spores (see Western analyses of spore proteins, above, for spore lysis protocol). Unbound *E. cuniculi* proteins were removed by washing in 1X TBST. The remaining bound *E. cuniculi* proteins were eluted in the presence of lysis buffer (see lysis buffer components above). The resulting protein sample was separated by SDS-PAGE. For Western analysis, membranes were incubated in a 1:500 dilution of the anti-rMsADAM antibodies followed by a 1:2000 dilution of goat anti-rabbit alkaline phosphatase-conjugated antibody (Southern Biotech; Birmingham, AL). BCIP and NBT were used for detection. The corresponding *E. cuniculi* protein band, located at ~143 kDa on the Coomassie stained SDS-PAGE gel, was excised and sent to Midwest Bio Services, LLC (Overland Park, KS) for mass spectrometric analysis.

**Immunolabeling and Transmission Electron Microscopy**

RK-13 monolayers grown in T 75 cm² flasks were incubated with *E. cuniculi* or *E. intestinalis* spores until numerous infectious foci were observed. Infected RK-13 monolayers were fixed for one hour with 2% paraformaldehyde, 0.05% glutaraldehyde in 0.2 M Sorenson’s buffer. Following several washes with 0.1 M Sorenson’s buffer, monolayers were removed by scraping and enrobed in 3% SeaKem agar. The agar-enrobed pellet was washed three times with 0.1 M Sorenson’s buffer at 4°C for 15 minutes. The pellet was sequentially dehydrated with 35%, 50%, and 70% methanol at 4°C for five minutes and with 90% methanol at -20°C for 30 minutes. The following infiltration steps were performed for one hour each at -20°C: 1:1 Lowicryl K4M resin/90% methanol, 2:1 Lowicryl K4M resin/90% methanol, and Lowicryl K4M resin only. The pellet was then incubated in Lowicryl K4M resin overnight at -20°C. The
pellet was embedded in Lowicryl K4M resin, and photopolymerization was carried out for two days at -20°C, two days at 4°C, and at room temperature for two days.

Sections were blocked in 1% albumin and 0.01 M glycine prepared in PBS for five minutes. These sections were then incubated with a 1:50 dilution of protein A/G purified polyclonal rabbit antibodies against rMsADAM in blocking solution for 40 minutes at 37°C and washed with PBS three times for five minutes each. Sections were blocked again for five minutes and incubated for 30 minutes at 37°C with a 1:200 dilution of AuroProbe EM 15 nm gold-labeled goat anti-rabbit IgG (H+L) (GE Healthcare; Piscataway, NJ). Sections were washed with PBS three times for five minutes followed by three 15 minute washes with sterile water. Sections were then counterstained with 5% uranyl acetate, washed with sterile water, and examined using a Tecnai 10 transmission electron microscope.

**Spore Adherence and Infection Assays**

Spore adherence assays were performed as previously described [5]. Briefly, Vero cells were seeded onto 18 mm² glass coverslips at a density of 1X10⁵ cells per well in 12 well plates with growth medium (see growth medium components above) and grown to confluence. For inhibition studies, the recombinant proteins rMsADAM, rPRO, rMET or rHsp70 (histidine-tagged recombinant microsporidia endoplasmic reticulum-associated heat shock protein [7,8]), and the above described polyclonal anti-rMsADAM antibodies and anti-rHsp70 antibodies were diluted in 1mM MnCl₂-supplemented growth medium at final concentrations of 0.0001 mcg ml⁻¹ to 1 mcg ml⁻¹ recombinant protein or 1:100 diluted antibodies.

To measure spore adherence, the 12 well plates containing the coverslips with Vero monolayers were placed on ice for 30 minutes. Following the incubation, the growth medium was removed from the 12 well plates, and one ml per well of the MnCl₂-supplemented media with 10⁷ *E. intestinalis* spores, and either recombinant proteins or
antibodies was added to the plates. The 12 well plates were then incubated on ice for four hours, and coverslips were washed with PBS to remove unbound spores. The Vero monolayers with attached spores were fixed with a 1:1 solution of acetone:methanol for 10 minutes at room temperature, washed by covering with PBS for five minutes and stained with 0.01% Uvitex 2B (Polysciences, Inc.; Warrington, PA) for 10 minutes. Samples were then washed with PBS for five minutes, counterstained with 1 mcg ml\(^{-1}\) 4',6-Diamidino-2-Phenylindole, Dichloride (DAPI) (Life Technologies Corporation; Carlsbad, CA) for five minutes, washed with PBS for five minutes and inverted onto VECTASHIELD mounting medium (Vector Laboratories; Burlingame, CA) on microscope slides. The number of attached spores and host cell nuclei were counted in 20 fields per coverslip at 630X (oil immersion) magnification with an ultraviolet filter using an Olympus BH-2 fluorescent microscope. Results for adherence are expressed as the percentage of adherent spores relative to control samples.

To measure host cell infection, spore adherence assays were performed as described above. However, following the PBS wash to remove unbound spores, the coverslips were placed into new 12 well plates with fresh growth medium and incubated in 5% CO2 at 37\(^\circ\)C for ~30 hours to allow the attached spores to infect the host cells. Fixing, staining and counterstaining were performed as described above. The number of infectious foci and host cell nuclei were counted in 20 fields per coverslip at 630X magnification using an ultraviolet filter. Results for host cell infection are expressed as percentage of infection (infectious foci per nucleus) relative to control samples.

For adherence and infection assays, experiments were performed three times with similar results. Data from representative experiments are shown. Statistical significance was determined using the Student’s \(t\) test and \(p\) values of <0.05 (*) and <0.0001 (**) were considered statistically significant and are indicated. Error bars represent standard deviation.
Results

Identification of an ADAM Family Member in Microsporidia

Putative proteins from the *E. cuniculi* database were screened for motifs potentially involved in adhesion by comparison to the Conserved Domain Database of the National Center of Biotechnology Information [22]. One protein (ECU06_0380) was identified as having motifs similar to both the disintegrin domain of snake venom proteins (expect value $6 \times 10^{-10}$) and the reprolysin domains of zinc metalloproteases (expect value 0.002). The primary structure of the protein has multiple domains that are similar to those of known ADAM family members (Figure 3.1), including a predicted signal peptide sequence, pro-domain, metalloprotease domain, disintegrin domain, and a transmembrane domain. Based on these sequence similarities, the protein has been named microsporidia ADAM (MsADAM). MsADAM differs from other ADAM family members because it apparently lacks a recognizable cysteine-rich domain and a cytoplasmic tail. Instead, between the disintegrin and transmembrane domains, MsADAM has an unknown domain of approximately 76 amino acids, which has no significant similarity to any other proteins.

Global pairwise alignment of MsADAM with other ADAM family members showed sequence identities ranging from 15.2% (ADAM 19) to 20% (ADAM 21). When comparing domains independently, the MsADAM disintegrin domain was most identical to its counterparts in other ADAMs (identity ranges from 28.1% (ADAM 4) to 36.5% (ADAMs 9 and 12)) (Figure 3.1). This higher level of identity is due in part to the conserved cysteine residues in the disintegrin domain and suggests that MsADAM may have a similar secondary structure and function to other ADAMs. Interestingly, MsADAM has a five amino acid insertion in the area proposed to be the integrin-binding region of the disintegrin domain [16]. The ability of microsporidia to bind to host cell integrins is unknown, but studies are currently underway to determine the nature of integrin/MsADAM interaction(s).
Figure 3.1 Schematic Representation of the MsADAM Domains Compared to the Consensus ADAM Domains. MsADAM shares with ADAM proteins a pro-domain, metalloprotease domain, disintegrin domain, and transmembrane domain. The sequence of the metalloprotease domain catalytic site and the disintegrin domain from several ADAM family members is shown in an alignment to illustrate that MsADAM has an apparent functional catalytic site, a conserved downstream cysteine residue indicative of the metzincin family of metalloproteases, and conserved cysteine spacing of the disintegrin domain. MsADAM also has a five amino acid insertion in the integrin binding loop (shown as a double over line).

The MsADAM metalloprotease domain contains the consensus sequence of an active endopeptidase (HEXXHXXGXXH). A methionine residue in close proximity to the active site of the metalloprotease domain places MsADAM in the metzincin group of endopeptidases [23]. The methionine residue in MsADAM is located seven amino acids away from the last histidine residue of the catalytic site, whereas the distance ranges from 14 to 23 amino acids in other ADAMs. Although enzymatic assays using
fibronectin, gelatin, azocasein and rMsADAM have been performed, the metalloprotease activity of MsADAM has yet to be established.

The pro-domain of an ADAM is thought to keep the endopeptidase in an inactive state. Removal of the pro-domain by proteolytic cleavage or autocatalysis activates the endopeptidase [24]. Some ADAMs use a cysteine-switch mechanism of inactivation in which a non-paired cysteine residue in the pro-domain interacts with the metalloprotease site, keeping it inactive. While these ADAMs have an odd number of cysteines in the pro-domain region, MsADAM has an even number of closely spaced cysteine residues, suggesting that it may not use the cysteine-switch mechanism of pro-domain inactivation. The MsADAM pro-domain also lacks a recognizable furin protease cleavage site, which is not unexpected because the *E. cuniculi* genome may not have a furin-like protease gene (personal observation). MsADAM may, however, be activated by different proteases, as proposed for other ADAMS that lack furin recognition sites. It is also possible that MsADAM is auto-catalytically activated in a manner similar to ADAM 28 [25].

Pairwise alignments of *E. cuniculi* MsADAM with three other *Encephalitozoon* species showed a high level of conservation between species, with sequence identities to *E. cuniculi* MsADAM ranging from 63.6% (*E. intestinalis*) to 66.5% (*E. hellem*) in human pathogens and 65.4% (*E. romaleae*) in an invertebrate pathogen. Interestingly, *E. hellem* and *E. romaleae* showed the highest level of identity, at 76.6%, among the four species despite their distinct host organisms. The metalloprotease domain, including the conserved active endopeptidase sequence (HEXXHXXGXXH), the disintegrin domain, including both the cysteine residues similar to those found in other ADAMs and the five amino acid insert not seen in other ADAMs, and cysteine residue numbers and spacing are particularly highly conserved among the *Encephalitozoon* species, suggesting that MsADAM may function similarly within these members of the genus. In this study, we used the *E. cuniculi* MsADAM sequence for recombinant
protein expression, and the spores of two *Encephalitozoon* species, *E. cuniculi*, and *E. intestinalis*, to investigate the potential role of MsADAM in spore adherence and host cell infection.

**Recombinant Expression, Polyclonal Antibody Production and Western Blot Analysis of MsADAM in Spores**

To characterize the MsADAM protein, a histidine-tagged recombinant MsADAM protein (rMsADAM) was expressed in *E. coli*, purified by nickel affinity chromatography, and used to generate antiserum. DNA encoding the region from the pro-domain to the transmembrane-domain of MsADAM (amino acids 21-527) was cloned into a histidine-tagging vector, expressed in *E. coli* and analyzed by SDS-PAGE and Western blot using a monoclonal anti-polyhistidine antibody. Upon induction, a protein of molecular weight ~61 kDa was expressed (consistent with the predicted size of ~59 kDa for the rMsADAM protein) and was specifically recognized by the anti-histidine antibody (Figure 3.2A). When rMsADAM-specific antibodies were purified from immunized rabbit serum and used in Western analyses of total spore protein lysates from both *E. cuniculi* and *E. intestinalis*, the anti-rMsADAM antibodies recognized proteins of ~60 kDa, which is approximately the predicted size of the full length MsADAM protein, and an unexpected protein species of ~143 kDa (Figure 3.2B).

**Identification of The ~143 kDa Protein Recognized by Anti-rMsADAM Antibodies**

To purify and identify the anti-rMsADAM-reactive ~143 kDa protein species, *E. cuniculi* spore protein lysate was incubated on nitrocellulose membrane that had been pre-incubated with the anti-rMsADAM antibodies. After washing the membrane to remove unbound proteins, *E. cuniculi* proteins that remained bound to the membrane were eluted and the resulting protein sample was separated by SDS-PAGE, Coomassie stained, and subjected to Western analysis using the anti-rMsADAM antibodies (Figure
Figure 3.2 Recombinant MsADAM Expression and Antibody Production. (A) Expression of recombinant MsADAM protein. Lysates from *E. coli* cultures were separated by SDS-PAGE and Coomassie stained (lanes 1-3) or processed for Western analysis (lanes 4-6) using anti-histidine antibody. Upon induction, a protein of ~61 kDa was observed (lane 3) and recognized by the anti-histidine antibody (lane 6). The protein, rMsADAM, was not observed in the non-induced *E. coli* lysate (lanes 2 and 5). Lanes 1 and 4 show molecular weight markers. (B) Western analyses with anti-rMsADAM antibodies revealed specificity for MsADAM and an additional protein. Lysates from *E. cuniculi* (lane 2) and *E. intestinalis* (lane 3) were processed for Western analysis. Anti-rMsADAM antibodies recognize proteins of ~60 kDa and ~143 kDa from both *E. cuniculi* and *E. intestinalis*. The *E. cuniculi* MsADAM protein is predicted to be ~61 kDa and ~59 kDa after signal sequence removal. Lane 1 shows molecular weight markers. (C) Purification and identification of the ~143 kDa *E. cuniculi* protein. Nitrocellulose membrane pre-incubated with anti-rMsADAM antibodies was incubated with *E. cuniculi* lysate. Unbound proteins were washed away, and bound proteins were eluted. The proteins were Coomassie stained (lane 2) or processed for Western analysis (lane 4) using the anti-rMsADAM antibodies. As seen in lanes 2 and 4 a ~143 kDa protein was isolated. This protein was excised from lane 2, subjected to mass spectrometric analysis, and identified as *E. cuniculi* polar tube protein 3. Lanes 1 and 3 show molecular weight markers. (D) Western analysis of spore protein using rabbit pre-immune serum. Lysates from *E. cuniculi* (lane 2) and *E. intestinalis* (lane 3) were processed for Western analysis with rabbit pre-immune serum. The serum recognized a protein of ~143 kDa in both spore lysates, but did not recognize the rMsADAM protein (lane 4). Lane 1 shows molecular weight markers.
3.2C). While no protein of ~60 kDa was eluted from the membrane, perhaps due to comparatively stronger interaction of MsADAM to the antibody-incubated membrane, several proteins, including a protein of ~143 kDa, were recovered from the membrane and recognized by the anti-rMsADAM antibodies. The corresponding ~143 kDa protein band was excised from the Coomassie stained gel, subjected to mass spectrometric analysis, and identified as \textit{E. cuniculi} polar tube protein 3 (PTP3) which has a predicted molecular weight of ~136 kDa and an apparent molecular weight of ~150 kDa upon SDS-PAGE [26].

Subsequent reexamination of the pre-immune rabbit serum revealed that serum from the animal used for antibody generation, collected prior to immunization with rMsADAM, contained antibodies reactive to several \textit{E. cuniculi} and \textit{E. intestinalis} proteins, including a species of ~143 kDa, but was not reactive to the rMsADAM protein (Figure 3.2D). Microsporidia, particularly \textit{E. cuniculi}, naturally infects rabbits, therefore finding an entirely naïve rabbit for antibody production may continue to be a challenge. In fact, Western analyses of several other pre-immune rabbit sera from the supplier used to generate our anti-rMsADAM serum and from additional suppliers demonstrated that the rabbits already generated antibodies that reacted to multiple \textit{E. cuniculi} and \textit{E. intestinalis} proteins of various sizes (data not shown). Future generation of more specific anti-rMsADAM antibodies will be attempted in rodents.

**Localization of MsADAM in \textit{E. cuniculi} and \textit{E. intestinalis} Spores**

To determine the location of MsADAM in spores, host cells were infected \textit{in vitro} with \textit{E. intestinalis} or \textit{E. cuniculi} spores, immunolabeled using either antibodies from pre-immune serum or purified anti-rMsADAM antibodies, and examined by transmission electron microscopy (TEM). In infected cells labeled with pre-immune antibodies, no host cell or spore structures were labeled in either \textit{E. cuniculi} infected host cells (data
not shown) or in *E. intestinalis* infected host cells (Figure 3.3). Because the pre-immune serum antibodies failed to label spore structures, including those specifically immunolabeled by the anti-rMsADAM antibodies, the reactivity of the pre-immune serum demonstrated in Western analyses should not impact the TEM localizations presented in this study. It is possible that the significant effects of detergents and reducing agents used to generate the spore lysates for SDS-PAGE exposed protein motifs in Western analyses that were not available for antibody binding in alternatively manipulated samples used for TEM.

![Figure 3.3 Immunolabeling of *E. intestinalis* and *E. cuniculi* Infected RK-13 Cells with Pre-immune Serum Antibodies.](image)

Figure 3.3 Immunolabeling of *E. intestinalis* and *E. cuniculi* Infected RK-13 Cells with Pre-immune Serum Antibodies. Sections of *E. intestinalis* or *E. cuniculi* infected RK-13 cells were labeled with antibodies from rabbit pre-immune serum followed by 15nm gold-labeled goat anti-rabbit secondary antibody. As seen in this representative image of *E. intestinalis* infected cells, no specific labeling was observed within the major spore structures including the polar sac (PS), anchoring disk (AD), polar tube (PT), plasma membrane (PM), endospore (EN), and exospore (EX). No labeling occurred within surrounding host cells, and no labeling of *E. cuniculi* spores was observed (data not shown).
In infected host cells labeled with the anti-rMsADAM antibodies, MsADAM localizes to the bell-shaped anchoring disk/polar sac region of both *E. intestinalis* and *E. cuniculi* spores (Figures 3.4 and 3.5). 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 sac and terminates in a biconvex disk shape at its center. This region helps anchor the polar filament to the spore wall and plays a role in the rupture of the spore wall and subsequent extrusion of the polar tube following spore activation [27]. Immunolabeling with anti-rMsADAM antibodies specifically and consistently localizes MsADAM expression to the flared biconvex anchoring disk region of both *E. intestinalis* and *E. cuniculi*; while MsADAM expression is comparatively sparse in the polar sac region of both species (Figures 3.4 and 3.5).

Outside of the anchoring disk/polar sac region, MsADAM expression in *E. cuniculi* and *E. intestinalis* differs significantly. In *E. cuniculi*, MsADAM is located only in the anchoring disk and polar sac (Figure 3.4). In *E. intestinalis*, however, MsADAM localizes not only to the anchoring disk/polar sac complex, but also to the exospore and plasma membrane (Figure 3.5). This difference in MsADAM expression is unexpected due to the high degree of MsADAM sequence conservation among the *Encephalitozoon* species. Because the *E. cuniculi* MsADAM sequence was used to express the protein for generating the antibodies used in this study, low anti-rMsADAM antibody affinity for *E. cuniculi* MsADAM compared to *E. intestinalis* MsADAM probably does not fully explain the relatively limited MsADAM labeling observed in *E. cuniculi*. Thus, the interspecific variation in MsADAM localization is of interest, and further examination of the nature of the variation may reveal biologically important differences between microsporidia species.
Figure 3.4 Localization of MsADAM in *E. cuniculi* Spores. Sections of *E. cuniculi* infected RK-13 cells were labeled with anti-rMsADAM antibodies followed by 15nm gold-labeled goat anti-rabbit secondary antibody. (A-B) Immunolabeling was observed within the polar sac (PS) and anchoring disk (AD) of *E. cuniculi* spores. (C) No significant immunolabeling was observed in the exospore (EX), endospore (EN), or plasma membrane (PM) of *E. cuniculi* spores. (D) Immunolabeling was observed within the anchoring disk (AD) in spores with extruded polar tubes. No labeling occurred within surrounding host cells.
Figure 3.5 Localization of MsADAM in *E. intestinalis* Spores. Sections of *E. intestinalis* infected RK-13 cells were labeled with anti-rMsADAM antibodies followed by 15nm gold-labeled goat anti-rabbit secondary antibody. (A-E) Immunolabeling was observed within the polar sac (PS), anchoring disk (AD), plasma membrane (PM), and exospore (EX), but not endospore (EN) of *E. intestinalis* spores. (D) Immunolabeling was observed within the anchoring disk (AD) in spores with extruded polar tubes. No labeling occurred within surrounding host cells.
Spore Adherence and Host Cell Infection Assays

To evaluate potential involvement of MsADAM in the adherence and infection process, a series of in vitro assays were performed to determine if the MsADAM protein, individual MsADAM domains, or anti-MsADAM antibodies could be used to manipulate spore adherence and host cell infection. In addition to the nearly full-length rMsADAM protein, which lacks only the signal sequence and transmembrane domains and was used for antibody production, we expressed two individual domains of the *E. cuniculi* MsADAM protein. rPRO consists of the pro-domain alone, while rMET consists solely of the MsADAM metalloprotease domain (Figure 3.6). Attempts to express the MsADAM disintegrin domain alone were unsuccessful. Microsporidia Hsp70, previously shown to be uninvolved in spore adherence [7,28], served as the control in the spore adherence and host cell infection assays.

Figure 3.6 Recombinant MsADAM Constructs Expressed for Use in Spore Adherence and Host Cell Infection Assays. The domains of the full-length MsADAM protein, based on sequence analysis, are shown (MsADAM), followed by constructs used to express multiple and individual domains of the protein for use in spore adherence and host cell infection assays. rMsADAM, the recombinant protein used for antibody production, lacks only the signal sequence (SS) and transmembrane (TM) domains, while rPRO and rMET consist of only the pro-domain and metalloprotease domain, respectively. All constructs are histidine-tagged and were expressed in *E. coli.*
adherence and infection assays. Recombinant Hsp70 (rHsp70), anti-rHsp70 antibodies, and protein diluent alone (used in protein serial dilutions) each had no effect on adherence or infection compared to assays carried out with no additional exogenous components (data not shown).

We have shown in previous studies that inhibiting spore adherence in vitro reduced subsequent infection of host cells [5]. In the present study, E. cuniculi adherence to host cells was not affected by the addition of exogenous rMsADAM to assays of spore adherence (data not shown) and infection assays with E. cuniculi were not conducted. In assays of E. intestinalis spore adherence to host cells, however, the addition of either exogenous rMsADAM or rMET inhibited spore adherence to host cells by ~20 to 30% of control adherence, while the addition of rPRO had no effect on adherence (Figure 3.7A). Serial dilutions of rMsADAM and rMET proteins added to spore adherence assays confirmed that these proteins exerted the observed inhibition of E. intestinalis adherence in a dose-dependent manner (Figure 3.7B). Addition of exogenous rMsADAM or rMET not only inhibited adherence of E. intestinalis to host cells, but also inhibited host cell infection by ~20% in comparison to control infection (Figure 3.7C). Addition of exogenous rPRO, which had no effect on spore adherence, did not affect host cell infection (Figure 3.7C). When anti-rMsADAM antibodies were added to assays of E. intestinalis adherence and infection of host cells, similar degrees of inhibition of adherence and infection (~ 20%) were observed in comparison to control Hsp70 antibodies, supporting the involvement of MsADAM in E. intestinalis adherence to and infection of host cells (Figure 3.7D).
Figure 3.7 Spore Adherence and Infection Assays. (A) Recombinant proteins (1 mcg ml\(^{-1}\)) were incubated on Vero monolayers in the presence of *E. cuniculi* or *E. intestinalis* spores. Monolayers were washed to remove unbound spores and evaluated for adherent spores. (B) Dilutions of rMsADAM and rMET (0.0001-1 mcg ml\(^{-1}\)) were incubated on Vero monolayers in the presence of *E. intestinalis*. Monolayers were washed and evaluated for adherent spores. (C) Recombinant proteins (1 mcg ml\(^{-1}\)) were incubated on Vero monolayers in the presence of *E. intestinalis*. Monolayers were washed and returned to culture to allow host cell infection then evaluated for infectious microsporidia. (D) Anti-rHsp70 and anti-rMsADAM antibodies (1:100 dilution) were incubated on Vero monolayers in the presence of *E. intestinalis*. Monolayers were evaluated for adherent spores and host cell infection. (A-D) Twenty microscopic fields were evaluated per coverslip in each of three experiments with similar results. Representative experiments are shown. Statistically significant differences from controls were determined using the Student’s *t* test and *p* values of <0.05 (*) and <0.0001 (**) were considered statistically significant and are indicated. Error bars represent standard deviation.
Discussion

The focus of our research is identifying host and microsporidia proteins that may be involved in spore adherence and host cell infection. Previously, we characterized EnP1, a ~40 kDa microsporidia protein with no homology to known proteins that may act as a spore adherence ligand [7, 8]. We showed that EnP1 is expressed in both *E. cuniculi* and *E. intestinalis* spores, and localizes to the polar sac/anchoring disk complex, endospore and exospore in both species. Studies using mouse anti-EnP1 antibodies [29], suggested that EnP1 is localized only to the microsporidia endospore and not the exospore. Nevertheless, addition of exogenous recombinant Enp1 or rabbit anti-EnP1 antibodies to spore adherence and infection assays inhibited the adherence of *E. cuniculi* and *E. intestinalis* to host cells and inhibited subsequent infection of these cells, supporting the spore surface expression of EnP1 [7]. MsADAM is similar to EnP1 in that both proteins localize to the exospore of *E. intestinalis* and to the polar sac/anchoring disk region of *E. intestinalis* and *E. cuniculi* spores. In contrast to EnP1, however, MsADAM does not localize to the endospore of either *Encephalitozoon* species or to the exospore of *E. cuniculi*. Additionally, EnP1 does not localize to the plasma membrane in either species; while MsADAM is located in the plasma membrane of both *Encephalitozoon* species. In view of these data, it is clear that the antibodies specific to either EnP1 or MsADAM recognize unique and specific proteins.

In the present study, a novel protein with a metalloprotease catalytic site, a disintegrin domain, and a transmembrane domain has been identified in microsporidia. The features of this protein suggest that it is a member of the ADAM family of zinc metalloproteinases, thus it has been named MsADAM (microsporidia ADAM). ADAM proteins are involved in a variety of functions including adhesion and proteolysis. Because MsADAM is found on the spore surface and in the anchoring disk complex, it may modulate either spore proteins or host cell surface proteins during the spore adherence and/or activation process that leads to spore germination and host cell
infection. MsADAM lacks a recognizable cysteine-rich domain, which may be important because the cysteine-rich domain and the disintegrin domain of ADAMs are thought to be responsible for the physical interaction of ADAMs with other proteins and their protease specificity [16]. In fact, crystallography studies of snake venom ADAMs have shown that these two domains are accessible for protein-protein interaction [30]. In place of the cysteine-rich domain, MsADAM has a notably shorter unknown domain (~76 amino acids compared to ~190 amino acids in the cysteine-rich domain of most ADAMs) that bears no similarity to any known functional domains. Interestingly, some fungal ADAM family members have shortened cysteine-rich domains (Magnaporthe grisea, accession XP_365884; Gibberella zeae, XP_391400; Aspergillus nidulans, XP_663223), but the MsADAM unknown domain shows no significant amino acid similarity to the shortened fungal ADAM cysteine-rich domains. The role of this unique undefined MsADAM domain and its possible effect on the disintegrin domain, may result in a function distinct from that of other ADAM proteins.

Previous analysis of the E. cuniculi genome suggested that microsporidia have relatively few known peptidases; however, the presence of metallopeptidases in soluble extracts of microsporidia spore precursor cells was suggested by proteolytic activity of the extract toward azocasein [19]. Four protein bands, ranging in size from 50 to 76 kDa, cleaved casein in zymogram gels. Two of these proteins were identified by mass spectrometric analysis as an aminopeptidase A and a leucine aminopeptidase. Other studies have shown that the fungal metabolite fumagillin targets a microsporidia methionine aminopeptidase-2 [31] and that the synthetic fumagillin analog TNP470 strongly inhibits recombinant methionine aminopeptidase [32]. MsADAM appears to be a metallopeptidase, but, despite our repeated attempts, specific proteolytic activity for MsADAM has not been established, even though the protein appears to have a functional catalytic site. It is possible that proper post-translational modifications are necessary for activity and that the recombinant MsADAM protein expressed in E. coli
does not have these characteristics. It is also possible that MsADAM metallopeptidase activity requires the presence of specific targets like ADAM 8, which actively cleaves the IgE surface receptor CD23 but not TNFα, which is cleaved by ADAM 17 [33]. Identifying target sequence(s) for MsADAM will be an essential part of deciphering its function.

Our TEM data localizes MsADAM to the flared funnel-shaped anchoring disk and the terminal polar sac of both *E. intestinalis* and *E. cuniculi* spores. The polar sac is a bell-shaped structure enclosed in a unit membrane at the apex of the spore. The anchoring disk is the flared biconvex terminal end of the coiled polar tube, which terminates at and is embedded in the polar sac. MsADAM may be anchored in these membranes via the protein's transmembrane domain. Although the function of MsADAM in this location is unknown, the involvement of this region in rupture of the spore that releases the evert ing polar tube suggests that MsADAM, a putative protease, may be involved in the activation process that initiates this rupture.

Importantly, the expression pattern of MsADAM in *E. intestinalis* is different than in *E. cuniculi*. MsADAM is located only in the polar sac/anchoring disk region in *E. cuniculi*. In *E. intestinalis*, MsADAM is located in the exospore, the plasma membrane, and the polar sac/anchoring disk region. The expression of MsADAM on the surface of *E. intestinalis* but not *E. cuniculi* is yet another indicator of the uniqueness *E. intestinalis* within the genus. *E. intestinalis* was originally classified as its own genus (*Septata intestinalis*) within the family *Encephalitozoonidae* [34] but was re-classified to the genus *Encephalitozoon* based on intergenic sequence data [35]. Another attribute that separates *E. intestinalis* from other species of this family is morphologic evidence of fibrillar material surrounding mature spores inside parasitophorous vacuoles [34]. The variation of protein expression patterns among microsporidia species gives a unique advantage to researchers. Because we cannot genetically manipulate microsporidia,
the observed differential localization of MsADAM in two *Encephalitozoon* species will be beneficial in studying differences in the function of this protein between species.

The interspecific variation in MsADAM expression raises interesting questions concerning the role MsADAM may play in microsporidia adherence to and infection of host cells. The lack of effect of exogenous rMsADAM on *E. cuniculi* adherence to host cells was not unexpected, considering the observed absence of MsADAM on the surface of *E. cuniculi* spores. The comparatively restricted localization of MsADAM in *E. cuniculi* does not eliminate the possibility that this protein may play a role in host cell infection by *E. cuniculi*. While surface MsADAM may act as a ligand for host cell surface receptor(s) involved in adherence and infection in *E. intestinalis*, it may have a different function in the polar sac/anchoring disc complex, where it is similarly located in both of the *Encephalitozoon* species. Because the polar sac/anchoring disc complex is involved in spore rupture and polar tube eversion, putative proteolytic activity of MsADAM may affect events that occur independently of spore adherence, yet still have an effect on subsequent infection of host cells.

In accordance with our demonstrated localization of MsADAM to spore surface structures in *E. intestinalis* spores, we showed that the addition of exogenous rMsADAM to spore adherence assays inhibited adherence of *E. intestinalis* to host cells. Variation in MsADAM expression, especially in the exospore, could impact host range, affect prevalence, or play a role in dissemination. Interestingly, although both species of microsporidia considered in the present study infect humans, *E. intestinalis* is more commonly identified in acquired immunodeficiency syndrome (AIDS) patients [36]. Nevertheless, any correlation of MsADAM exospore expression in *E. intestinalis* to infection of humans or other animals *in vivo*, while intriguing, remains to be confirmed.

Exogenous rMsADAM and rMET inhibited *E. intestinalis* spore adherence to the same extent. The rPRO domain, however, failed to inhibit spore adherence. These data suggest that the metalloprotease domain of MsADAM is the component
responsible for the observed inhibition of spore adherence and that the remaining
domains or, perhaps, the overall structure of the protein, are not required for this aspect
of MsADAM function and activity. However, examination of the MsADAM disintegrin
domain, which we were unfortunately unable to independently express and purify, would
reveal information about the role that this domain may play in microsporidia adherence
and infection, especially concerning possible interaction with host cell integrins.

Similar to our previous studies of spore and host cell determinants of adherence
and infection [5–7], the observed rMsADAM- and rMET-dependent inhibition of
adherence in the present study correlated with a decrease in subsequent host cell
infection. Thus the data presented in this study support our hypothesis that spore
adherence is an integral event in the infection of host cells by microsporidia. It is
notable that the inhibitory effect of exogenous rMsADAM (or anti-rMsADAM) on in vitro
adherence and infection was demonstrated only in microsporidia with surface
expression of MsADAM (E. intestinalis, but not E. cuniculi). A previous study
demonstrated a similar inhibitory effect of exogenous recombinant EnP1 (or anti-EnP1
antibodies) on in vitro adherence and infection in both Encephalitozoon species, in
correlation with EnP1 surface expression in both species.

As for many infectious microorganisms, the process of microsporidia adherence
and infection is complex and may involve multiple parasite and host proteins. In this
study we have identified potential microsporidia ADAM (MsADAM) proteases in E.
intestinalis and E. cuniculi. Immunoelectron microscopy of host cells infected with E.
cuniculi in vitro localizes MsADAM only to the polar sac/anchoring disk complex of E.
cuniculi spores. No evidence for involvement of MsADAM in E. cuniculi adherence and
infection was demonstrated. In contrast, immunoelectron microscopy of host cells
infected with E. intestinalis in vitro localizes MsADAM to the plasma membrane and the
exospore in addition to the polar sac-anchoring disk region of E. intestinalis spores.
Spore adherence and infection assays were used to demonstrate an inhibitory effect of
exogenous rMsADAM on adherence and infection of *E. intestinalis*, but not *E. cuniculi* spores. Our data thus suggest that surface MsADAM is involved in *E. intestinalis* adherence and infection of host cells. Because MsADAM has the potential to act as a metalloprotease and/or disintegrin, further studies of the protein may reveal additional activity or interactions that affect the infection process of the *Encephalitozoon* species.

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References


CHAPTER 4

ROLE OF HOST CELL INTEGRINS IN ENCEPHALITOZOOON INTESTINALIS
ADHERENCE AND INFECTION

Cory A. Leonard and J. Russell Hayman*

Department of Biomedical Sciences, East Tennessee State University, James H. Quillen College of Medicine, Johnson City, TN 37614

*Corresponding author: Mailing address: ETSU/JHQ-COM, Department of Microbiology, Box 70577, Johnson City, TN 37614. Phone: (423) 439-6313. Fax: (423) 439-8044. Email: hayman@etsu.edu

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Abstract

Microsporidia are obligate intracellular, spore-forming, fungal-related pathogens which employ a unique organelle, the polar tube, to transfer infectious spore contents into host cells to initiate infection. Microsporidia spores adhere to host cell surfaces \textit{in vitro} and it is likely that during \textit{in vivo} infection, spore adherence to host cells provides the proximity required for polar tube/host cell interaction. Determination of host cell and spore components that participate in adherence has begun to elucidate the role spore adherence plays in microsporidia infection of host cells \textit{in vitro}. In previous \textit{in vitro} studies, the addition of exogenous sulfated glycosaminoglycans (GAGs) or spore wall and anchoring disk complex protein EnP1 (EnP1) inhibited both spore adherence to host cells and subsequent infection of these cells by ~60 to 90%. Complete ablation of spore adherence and infection could not be achieved using sulfated GAGs or Enp1 as binding competitors suggesting that additional or alternative spore and host cell determinants of adherence and infection may exist. Analysis of the \textit{E. intestinalis} genome revealed over 50 predicted proteins containing the canonical integrin-binding motif and one predicted protein with a motif similar to the disintegrin domain of snake venom proteins, which are known to bind integrins. We therefore hypothesized that host cell integrins play a role in microsporidia adherence and infection. In this study, we demonstrated that the addition of exogenous integrin ligands or recombinant $\alpha3\beta1$ or $\alpha5\beta1$ integrins significantly reduced \textit{E. intestinalis} spore adherence and infection of host cells by ~60%, supporting our hypothesis and implicating these specific integrins as putative host cell receptors for \textit{E. intestinalis} spores.
Introduction

Microsporidia are obligate intracellular, spore-forming, fungal-related pathogens that infect a wide range of invertebrate and vertebrate animals. Of the fourteen species of microsporidia known to infect humans, *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis* are most commonly associated with human disease [1]. Immunocompromised individuals, such as those with acquired immune deficiency syndrome (AIDS) or those undergoing immune suppressive therapies following organ transplants or for the treatment of rheumatoid arthritis, can exhibit severe manifestations of microsporidiosis including chronic diarrhea, malabsorption, wasting, disseminated disease and death [3–5]. Immunocompetent individuals also show evidence of microsporidia infections, frequently with mild or no symptoms, suggesting that human exposure to microsporidia may be relatively common [6–8]. Currently available therapies for human microsporidiosis are extremely limited, and an understanding of the events and mechanisms of the infectious process of microsporidiosis is needed to facilitate development of safe and effective treatments for microsporidia infection.

Outside of the host, microsporidia exist as metabolically inactive, environmentally resistant spores [1]. The spore wall of microsporidia, consisting of the chitinous endospore and the protein-containing exospore, affords the spore rigidity and protection and also positions spore wall proteins to interact with host cell surface molecules. It is likely that microsporidia such as *E. bieneusi* and *E. intestinalis* are transmitted to humans via the fecal-oral route upon the ingestion of contaminated water [5]. Not surprisingly, given this route of infection, the initial site of infection in humans is usually the small intestine epithelium [9]. Inside the host, spores become activated and forcibly extrude a unique organelle, the polar tube, which may either pierce the host cell or be taken into the cell by cellular plasma membrane invagination [10]. The infectious material of the spore, the sporoplasm, passes through the polar tube into the cytoplasm of the host cell. Spores proliferate within the host cells, progressing through multiple
developmental stages before the host cells rupture. Newly released, mature spores, can infecting nearby cells, causing disseminated infection within the host, or passing into the environment.

Spores adhere tightly to host cell surfaces in vitro and cannot be removed by repeated washing [11]. It is likely that during in vivo infection, spore adherence to host cells is important to ensure the necessary proximity for polar tube/host cell interaction and the transfer of sporoplasm into the host cell. Determination of host cell and spore components that participate in adherence has helped clarify the role spore adherence plays in microsporidia infection of host cells in vitro. Previously, we have shown that in vitro spore adherence to host cells was significantly reduced by the addition of exogenous sulfated glycosaminoglycans (GAGs) and in cells that were deficient in surface sulfated proteoglycan expression, suggesting that endogenous cell surface sulfated GAGs are involved in spore adherence [11].

Additionally, we showed that the microsporidia spore wall and anchoring disk complex protein EnP1 (EnP1) is able to bind to host cells and that the addition of exogenous recombinant EnP1, or EnP1 antibodies, significantly inhibited spore adherence by 60% [12]. Importantly, both of these studies demonstrated that decreased spore adherence to host cells resulted in subsequent reduction in infection of these cells [11, 12]. These data helped shape our hypothesis that spore adherence is a critical part of microsporidia infection of host cells. Because complete ablation of spore adherence and infection was not achieved using exogenous sulfated GAGs or EnP1, additional or alternative spore and host cell determinants of adherence and infection may exist. To better understand the mechanism of microsporidia adherence to and infection of host cells, we have focused on determining which additional host cell surface proteins may be involved in this process.

Many pathogenic microbes including viruses, bacteria and parasites use mammalian cell surface proteoglycans (GAG chains attached to core proteins) and
extracellular matrix (ECM) proteins, as well as the integrins, which bind ECM proteins, as a means for adherence to and invasion of host cells, for spreading to neighboring cells and for dissemination within the host [13–16]. Integrins are heterodimeric, trans-membrane proteins which, through their interactions with ECM and cytosolic components carry out crucial functions in cell structure, development, adhesion, migration, and signaling [17].

The ECM proteins that serve as ligands for integrins have additional binding sites which allow interactions with other ECM proteins as well as with ECM carbohydrates [18]. ECM components are widely expressed on metazoan cells and form the interstitial matrix between cells and, via integrin attachments, anchor epithelial cells tightly to the basement membrane which separates epithelia from tissues underneath [18]. The human-infecting microsporidia are capable of infecting a range of animal hosts and host cell types, as is exemplified by the ability of *E. intestinalis* to infect multiple hosts and to disseminate to many organs within the human body [19, 20], so it may be inferred that various and/or widely distributed host cell surface moieties are involved in the microsporidia adherence and infection process.

The possible roles of host cell integrins in microsporidia spore adherence and infection of host cells *in vitro* were examined in this study. Analysis of the *E. intestinalis* database by comparison to the Conserved Domain Database of the National Center of Biotechnology Information [21] revealed over 50 predicted proteins containing the arginine-glycine-aspartic acid (RGD) amino acid sequence (the canonical integrin-binding motif) and one predicted protein (ECU06_0380) with a motif similar (expect value 6e-10) to the disintegrin domain of snake venom proteins, which are known to bind integrins [22, 23]. Consequently, host cell integrins were hypothesized to be involved in microsporidia adherence and infection of host cells, and the addition of exogenous integrins or integrin ligands to *in vitro* adherence and infection assays was hypothesized to inhibit spore adherence/infection. Data confirm that addition of
exogenous ECM integrin ligands or recombinant α3β1 or α5β1 integrins to assays of *E. intestinalis* adherence and infection significantly reduced spore adherence and infection of host cells, supporting our hypothesis.

**Materials and Methods**

**Host Cell Cultivation**

Adherent host cell lines including African green monkey kidney cells (Vero; ATCC CCL-81), Chinese hamster ovary cells (CHO-K1; ATCC CCL-61), and mutant Chinese hamster ovary cells deficient in heparan sulfate synthesis (CHO PgsD-677; ATCC CRL-2244) or deficient in glycosaminoglycan synthesis (CHO PgsA-745; ATCC CRL-2242) were used for microsporidia cultivation and/or spore adherence and host cell infection assays. The cell lines were cultivated as previously described [24] at 37°C and 5% CO₂ in culture medium consisting of Dulbecco’s modified Eagle’s medium (BioWhittaker; Walkersville, MD) supplemented with 2mM L-glutamine, 100U ml⁻¹ penicillin, 100U ml⁻¹ streptomycin, 0.25 mcg ml⁻¹ Amphotericin B and 10% fetal bovine serum (FBS). Vero cells, but not CHO-K1 or the corresponding mutant cell lines, were maintained in the above culture media with 2% FBS, instead of 10% FBS.

**Microsporidia Propagation and Purification**

*Encephalitozoon intestinalis* spores were propagated in Vero cells as previously described [24]. Briefly, cultures were maintained at 37°C in 5% CO₂ in culture medium supplemented with 2% FBS. Subconfluent Vero monolayers grown in 75 cm² tissue culture flasks were incubated with spores for 10-12 days with culture medium replaced as necessary. After collection of infected host cells debris in culture medium, spores were harvested by centrifugation at 3500 RPM (10 minutes) and purified from the host cell debris by washing with 0.25% sodium dodecyl sulfate (SDS) and several washes in
sterile water. Spores stocks were counted using a hemacytometer and stored in sterile water at 4°C.

**Spore Adherence Assay**

Spore adherence assays were performed as previously described [11], with the addition of exogenous protein or antibodies to the assay as putative inhibitors of spore adherence. Briefly, host cells were seeded onto 18 mm² glass coverslips, at 1X10⁵ cells per well density, in 12 well plates with 2% FBS-supplemented culture media and grown to ~95% confluence. To measure spore adherence, the 12 well plates containing the coverslips with Vero monolayers were incubated on ice for 30 minutes. Following this incubation, the culture medium was aspirated from the 12 well plates, and 10⁷ spores per well in 1 mM MnCl₂-supplmented culture media (MnCl₂ supplementation increases spore adherence [11]) was added to the plates. The 12 well plates were then incubated on ice for four hours, and coverslips were washed by dunking 10X in phosphate buffer pH 7.4 (PBS) to remove unbound spores.

The host cell monolayers with attached spores were fixed with a 1:1 solution of acetone:methanol for 10 minutes at room temperature, washed by covering with PBS for five minutes and stained with 0.01% Uvitex 2B (Polysciences, Inc.; Warrington, PA) for 10 minutes. The cells were then washed with PBS for five minutes, counterstained with 1 mcg ml⁻¹ 4’,6-Diamidino-2-Phenylindole, Dichloride (DAPI) (Life Technologies Corporation; Carlsbad, CA) for 5 minutes, washed with PBS for five minutes and inverted onto VECTASHIELD mounting medium (Vector Laboratories; Burlingame, CA) on microscope slides. The number of attached spores and host cell nuclei were counted at 630X (oil immersion) magnification with an ultraviolet filter using an Olympus BH-2 fluorescent microscope. Results for adherence assays are expressed as the percentage of adherent spores relative to control samples.
To evaluate the effect of exogenous integrin ligands on spore adherence to Vero cells, the extracellular matrix (ECM) proteins type 1 collagen (Southern Biotech; Birmingham, AL; product # 1200-03), laminin (BD Biosciences; San Jose, CA; product # 354232), plasma-derived fibronectin (Sigma-Aldrich; St. Louis, MO; product # F1141) and plasma-derived vitronectin (Sigma-Aldrich; product # V8379), the control protein bovine serum albumin (BSA; Sigma-Aldrich; product # A-7030), or cyclical RGD or RAD peptides (Peptides International; Louisville, KY; product # ICA-4304-V and product # PCA-3618-P1) were diluted in PBS (0.0001 to 10 mcg ml\(^{-1}\) concentrations) and pre-incubated with the host cells before spore adherence assays were performed. After the plates and cells were incubated on ice 30 minutes, the culture medium was aspirated, the cells were washed with PBS, and the proteins in PBS (or PBS alone, as a control) were incubated on the cells, on ice, for one hour. After pre-incubation, the PBS with proteins was aspirated from the cells, and spores in MnCl\(_2\)-supplemented culture media were incubated on the cells as described above.

To determine the effect of combined exogenous ECM proteins, as well as the effect of ECM pre-incubation in conjunction with the addition of exogenous glycosaminoglycan (chondroitin sulfate A (CSA); Sigma-Aldrich; product # C-8529) during spore adherence assays, collagen, laminin, fibronectin and vitronectin were combined at a concentration of 1 mcg ml\(^{-1}\) each (4 mcg ml\(^{-1}\) total protein concentration; combined ECM) in PBS and used for the one hour pre-treatment both with and without the addition of 10 mg ml\(^{-1}\) CSA to the spores in MnCl\(_2\)-supplemented culture media for adherence assays as described above. Control and CSA-only samples were pre-treated with PBS alone, before spore adherence assays with and without added CSA were performed.

To compare the effect of exogenous combined ECM proteins and RGD and RAD peptides on spore adherence in cell lines with differing cell surface glycosaminoglycan expression, CHO-K1, CHO pgsD-677 (deficient in heparan sulfate synthesis) and CHO
pgsA-745 cells (deficient in glycosaminoglycan synthesis) were used in spore adherence assays as described above. Due to CHO cell sensitivity to cold, PBS pre-incubation and MnCl$_2$ supplementation, the spore adherence assay was modified, for these three cell lines only, as follows: incubations were carried out at room temperature, proteins and peptides were diluted in serum-free culture media for pre-incubation of cells (pre-incubation with serum free media alone was used for control samples), and MnCl$_2$ was not added to the 2% FBS-supplemented culture media for spore incubation on host cells.

The effect of anti-integrin antibodies on spore adherence to Vero cells was evaluated by pre-incubation of the cells (one hour, on ice) with antibodies to $\alpha$1-6, $\alpha$v or $\beta$1-5 integrin chains (Millipore; Billerica, MA; product # ECM430 and product # ECM440) diluted in 2% FBS-supplemented culture medium to 5 or 20 mcg ml$^{-1}$ final concentration. After pre-treatment, the medium with antibodies was aspirated from the cells, and spores in MnCl$_2$-supplemented culture medium were incubated on the cells as described above. The effect of exogenous recombinant human integrins on spore adherence to Vero cells was evaluated by including 5 or 20 mcg ml$^{-1}$ final concentration PBS-diluted recombinant $\alpha$3$\beta$1 integrin, $\alpha$5$\beta$1 integrin, $\alpha$6$\beta$4 integrin (R&D Systems; Minneapolis, MN; product # 2840-A3-050, product # 3230-A5-050 and product # 5497-A6-050) or PBS alone, as a control, in the spores/MnCl$_2$-supplemented culture medium for adherence assays as described above. The Vero cells for the recombinant integrin spore adherence assays were not pre-incubated prior to the incubation of spores on the cells, because the recombinant integrins were included in the spores/MnCl$_2$-supplemented culture medium.

**Host Cell Infection Assay**

To measure host cell infection, spore adherence assays with putative inhibitors of spore adherence were performed as described above. However, following the PBS
wash to remove unbound spores, the coverslips were placed into new 12 well plates with fresh culture medium and incubated with 5% CO₂ at 37°C for ~30 hours to allow the attached spores to infect the host cells. Following incubation for infection, fixation, staining and counterstaining were performed as described for adherence assays above. The number of infectious foci and host cell nuclei were counted at 630X (oil immersion) magnification with an ultraviolet filter. Results for infection assays are expressed as percentage of host cell infection (infectious foci per nucleus) relative to control samples.

**Statistical Analysis**

For adherence and infection assays, 20 microscopic fields were evaluated per coverslip. Experiments were performed three times with similar results. Representative experiments are shown. Statistically significant differences from controls were determined using the Student’s t test and p values of <0.05 (*) and <0.0001 (**) were considered statistically significant. Error bars represent standard deviation.

**Results**

**Exogenous Extracellular Matrix Proteins and an RGD Peptide Inhibit Spore Adherence and Host Cell Infection**

Previous studies indicate that while up to 85% of microsporidia spore adherence to host cells can be inhibited by the addition of sulfated GAGs in a spore adherence assay, at least 15% of spores still adhere to host cells and lead to subsequent host cell infection under these conditions [11]. This suggests that sulfated GAG-mediated adherence to host cells is not the only mechanism of adherence microsporidia employ in the infectious process. A spore adherence and infection assay developed in our lab [11] was used to evaluate the possible role of integrins in *Encephalitozoon intestinalis* adherence to Vero host cells (an adherent African green monkey-derived cell line). The ECM proteins used as integrin ligands, and thus potential inhibitors of spore adherence,
include collagen, laminin, fibronectin, and vitronectin and have been shown to directly bind integrins [25].

Host cell pre-incubation with 1 mcg ml\(^{-1}\) collagen, laminin, fibronectin or vitronectin prior to addition of *E. intestinalis* spores significantly inhibited spore adherence to host cells by \(\sim\)35\% of control adherence (Figure 4.1A). When cells pre-
incubation of host cells prior to assay of *E. intestinalis* adherence and infection had no effect on spore adherence or subsequent host cell infection (Figures 4.1C and 4.1D). Pre-incubation of cells with serial dilutions of ECMs demonstrated that ECM-dependent inhibition of spore adherence to host cells is dose-dependent up to 1 mcg ml\(^{-1}\) (Figure 4.2). Pre-incubation of cells with higher concentrations of ECM proteins up to 10 mcg ml\(^{-1}\) did not result in further increased inhibition of adherence or infection (data not shown).

**Figure 4.2 Extracellular Matrix Protein-dependent Inhibition of *E. intestinalis* Adherence is Dose-dependent.** Vero monolayers were pre-incubated with serially diluted extracellular matrix proteins collagen, laminin, fibronectin or vitronectin in PBS (0.0001 to 1 mcg ml\(^{-1}\) final concentrations), or PBS alone prior to addition of *E. intestinalis* spores, washed to remove unbound spores and evaluated for adherent spores. Statistically significant differences from controls were determined using the Student’s *t* test and *p* values of <0.05 (*) and <0.0001 (**) were considered statistically significant and are indicated. Error bars represent standard deviation.

Because each ECM evaluated contains the canonical integrin-binding arginine-glycine-aspartic acid RGD amino acid sequence [18] and over 50 predicted *E. intestinalis* proteins contain this sequence, interaction of the RGD sequence of *E. intestinalis* spores with a host cell surface moiety is plausible. Short RGD-containing peptides have been shown to inhibit cell adherence to collagen, laminin, fibronectin, or vitronectin-coated culture plates [26] and to interact directly with integrins [18]. To determine if the RGD sequence alone can modulate spore adherence to host cells, cells were pre-incubated with a short RDG-containing peptide prior to assays of *E. intestinalis* adherence and infection.
Host cell pre-incubation with the RGD peptide, but not a control RAD peptide, significantly inhibited spore adherence to host cells in a dose-dependent manner, with inhibition of ~40% of control adherence at 10 mcg ml⁻¹ peptide concentration (Figure 4.3A). When cells pre-incubated with the RGD or RAD peptides were assayed for infection, host cell infection was significantly reduced by ~20% of control infection with 10 mcg ml⁻¹ RGD pre-incubation, while RAD pre-incubation had no effect on infection (Figure 4.3B). In vitro Inhibition of *E. intestinalis* adherence and infection of cells by pre-incubation of the cells with known integrin ligands, including both ECM protein ligands and the RGD peptide, supports our hypothesis that integrins are involved in microsporidia adherence and infection of host cells.

**Figure 4.3 RGD Peptide Inhibits *E. intestinalis* Adherence and Infection of Host Cells.** (A) Vero monolayers were pre-incubated with serially diluted RGD or RAD peptides in PBS (0.001 to 10 mcg ml⁻¹ final concentrations), or PBS alone prior to addition of *E. intestinalis* spores, washed to remove unbound spores and evaluated for adherent spores. (B) Vero monolayers were pre-incubated with 10 mcg ml⁻¹ of RGD or RAD peptides in PBS, or PBS alone prior to addition of *E. intestinalis* spores, washed to remove unbound spores, returned to culture to allow subsequent infection and evaluated for infected host cells. (A-B) Statistically significant differences from controls were determined using the Student’s *t* test and *p* values of <0.05 (*) and <0.0001 (**) were considered statistically significant and are indicated. Error bars represent standard deviation.

**Combined ECM proteins do not further inhibit adherence and infection compared to individual ECM proteins**

The approximately equivalent inhibition of *E. intestinalis* adherence to and infection of host cells (~35% and ~30%, respectively; Figures 4.1A and 4.1B) by 1 mcg
ml\(^{-1}\) of the individual ECM proteins evaluated suggested that these proteins might be operating via a common mechanism to elicit the observed effect on spore adherence/infection. To determine if the ECM proteins act via a common mechanism or via discrete mechanisms, combined ECM proteins were used to pre-incubate cells prior to assays of adherence and infection. Pre-incubation of cells with a combination of 1 mcg ml\(^{-1}\) each of collagen, laminin, fibronectin and vitronectin (4 mcg ml\(^{-1}\) total ECM proteins) significantly inhibited subsequent *E. intestinalis* adherence to and infection of host cells by ~40% and ~30%, respectively (Figures 4.4A and 4.4B). The failure of

Figure 4.4 Combined Extracellular Matrix Proteins, Alone or with Glycosaminoglycan, Fail to Further Inhibit Adherence and Infection. (A) Vero monolayers were pre-incubated with the combined extracellular matrix proteins collagen, laminin, fibronectin or vitronectin in PBS (1 mcg ml\(^{-1}\) final concentrations of each ECM, 4 mcg ml\(^{-1}\) final concentration of total ECMs; combined ECM), or PBS alone. After pre-incubation, addition of *E. intestinalis* spores was carried out in the presence or absence of 10 mg ml\(^{-1}\) CSA. Monolayers were washed to remove unbound spores and evaluated for adherent spores. (B) Vero monolayers were pre-incubated with the combined ECM in PBS, or PBS alone. After pre-incubation, addition of *E. intestinalis* spores was carried out in the presence or absence of 10 mg ml\(^{-1}\) CSA. Monolayers were washed, returned to culture to allow subsequent infection and evaluated for infected host cells. (A-B) Statistically significant differences from controls were determined using the Student’s *t* test and *p* values of <0.05 (*) and <0.0001 (**) were considered statistically significant and are indicated. Error bars represent standard deviation.

multiple, combined ECM proteins to inhibit spore adherence and infection to a greater extent than the individual ECM proteins alone, suggests that these proteins act with
redundancy via a common mechanism, as opposed to exhibiting additive or synergistic effects which would suggest non-redundant activity via different mechanisms.

Pre-incubation of Cells with ECM Proteins Prior to GAG-dependent Inhibition of Adherence and Infection does Not Further Decrease Adherence or Infection

Previous studies indicate that in assays of spore adherence and host cell infection, the addition of exogenous sulfated GAGs concomitantly with spores during spore adherence to host cells significantly inhibits both spore adherence to and infection of host cells [11]. Cells were pre-incubated with combined ECM proteins prior to assays of *E. intestinalis* adherence carried out in the presence of the exogenous sulfated GAG chondroitin sulfate A (CSA) to determine if ECM pre-incubation has an effect on sulfated GAG-dependent inhibition of adherence and infection. The concomitant addition of 10 mg ml\textsuperscript{−1} CSA with addition of spores to host cells significantly inhibited adherence by ~60\% of control adherence and significantly inhibited host cell infection by ~40\% of control infection (Figures 4.4A and 4.4B). Pre-incubation of cells with combined ECM proteins prior to spore adherence and infection assays with CSA did not increase inhibition of adherence or infection of host cells compared to control pre-incubation alone prior to the addition of spores and CSA (Figures 4.4A and 4.4B). These data suggest that the known sulfated GAG-mediated spore interaction with host cells and the ECM-mediated effect on spore adherence and infection demonstrated in this study may exert their effects via a single, perhaps complex and multi-component, mechanism.

ECM Proteins and the RGD Peptide do Not Inhibit *Encephalitozoon intestinalis* Adherence to Glycosaminoglycan-deficient Cells

In interesting examples of pathogen/host cell surface interaction, several invasive bacterial species, including *Yersina*, *Neisseria* and *Helicobacter* species, are capable of recruiting host sulfated GAGs. These bacterially-recruited GAGs then facilitate the
interaction of the GAG/bacteria complex with host cell surface vitronectin or fibronectin resulting in augmented bacterial adherence to host cells [27]. A series of assays was conducted to determine if the herein observed ECM-dependent inhibition of spore adherence and infection of host cells is dependent on host cell glycosaminoglycan composition. CHO-K1 cells (an adherent Chinese hamster ovary-derived cell line), CHO PgsD-677 cells (CHO mutant, deficient in heparan sulfate synthesis) and CHO PgsA-745 (CHO mutant, deficient in all glycosaminoglycan synthesis) [28,29] were pre-incubated with the combined ECM proteins, RGD peptide or RAD peptide prior to addition of *E. intestinalis* spores. The combined ECM proteins and RGD peptide, but not RAD peptide, significantly inhibited spore adherence to CHO-K1 cells, inhibiting adherence by ~40% and ~30%, respectively (Figure 4.5). Combined ECM proteins, 

![Figure 4.5 Extracellular Matrix Proteins and RGD Peptide do Not Inhibit *E. intestinalis* Adherence to Glycosaminoglycan-deficient Cells.](image)

CHO-K1, CHO PgsD-677 (deficient in heparan sulfate synthesis) and CHO PgsA-745 (deficient in glycosaminoglycan synthesis) monolayers were pre-incubated with the combined extracellular matrix proteins collagen, laminin, fibronectin or vitronectin in serum free culture medium (1 mcg ml\(^{-1}\) final concentrations of each ECM, 4 mcg ml\(^{-1}\) final concentration of total ECMs; combined ECM), RGD or RAD peptides in serum free culture medium (10 mcg ml\(^{-1}\)) or serum free culture medium alone prior to addition of *E. intestinalis* spores, washed to remove unbound spores and evaluated for adherent spores. Statistically significant differences from controls were determined using the Student’s *t* test and *p* values of <0.05 (*) and <0.0001 (**) were considered statistically significant and are indicated. Error bars represent standard deviation.

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RDG peptide or RAD peptide, however, failed to inhibit spore adherence to heparan sulfate-deficient CHO PgsD-677 cells or GAG-deficient CHO PdsA-745 cells (Figure 4.5). The effect of ECM proteins or RGD and RAD peptides on CHO-K1 or mutant CHO host cell infection was not determined. These data indicate that the herein described ECM protein- and RGD peptide-dependent inhibition of *E. intestinalis* adherence to monkey-derived Vero cells also occurs in hamster-derived CHO-K1 cells which produce both heparan sulfate and chondroitin sulfate. The same effect cannot be observed in CHO mutants deficient in glycosaminoglycan synthesis, which suggests that in at least some cell lines, exogenous ECM protein and RGD-dependent inhibition of adherence is also GAG-dependent.

**Specific Integrin Antibodies and Recombinant Integrins Inhibit Spore Adherence and Host Cell Infection**

The integrin ligand ECM proteins used in this study can directly interact not only with integrins, but also with other protein and carbohydrate components of the ECM as well [18]. Thus, the inhibition of spore adherence and infection by the addition of exogenous integrin binding partners to adherence/infection assays suggests host cell integrins are involved in spore adherence *in vitro*, but does not demonstrate that the exogenous ligands are interacting directly with integrins to exert the observed effects. Data generated using commercially available α and β integrin-mediated cell adhesion arrays (Chemicon (Millipore), per manufacturer’s instructions) indicated that the Vero cells used for spore adherence and infection assays in this study consistently bound anti-α3, α5, β1 or β2 integrin chain antibody-coated microplate wells, variably and poorly bound anti-α4 integrin chain antibody-coated wells, and failed to bind wells coated with other anti-integrin chain antibodies (data not shown). This indicates that α3,
α5, β1, β2 and possibly β4 integrin chains are expressed on the host cells used in our assays.

To more directly examine the possible role of host cell integrins in spore adherence and infection, Vero host cells were pre-incubated with 5 or 20 mcg ml⁻¹ commercially available (Chemicon (Millipore)) monoclonal, function-blocking antibodies against human α1-6, αv, or β1-5 integrin chains prior to assays of *E. intestinalis* adherence and infection. Pre-incubation with 20 mcg ml⁻¹ anti-α3, α5, β1 or β2 integrin chain antibodies only, but not the remaining antibodies, significantly inhibited spore adherence to host cells by ~30% of control adherence (Figure 4.6A). Adherence assays performed after similar pre-incubation of host cells with 5 mcg ml⁻¹ anti-integrin chain antibodies showed similar results without significantly less inhibition of adherence (data not shown), and possible dose-dependence of the inhibition of adherence was not further evaluated. Host cell pre-incubation with antibodies against α3, α5, β1 and β2 integrin chains also significantly inhibited infection of host cells by ~20% of control infection, while the remaining anti-integrin chain antibodies had no effect on infection (Figure 4.6B). The inhibition of spore adherence/infection of host cells by specific anti-integrin chain antibodies provides further, more direct support for our hypothesis that host cell integrins are involved in microsporidia adherence and infection of host cells.

Assays were conducted to determine if the addition of exogenous recombinant human α3β1, α5β1, or α6β4 integrins concomitantly with *E. intestinalis* spores during adherence/infection assays modulates spore adherence to and/or infection of host cells. The addition of α3β1 or α5β1 integrins to adherence assays significantly inhibited spore adherence by ~60% of control adherence (Figure 4.7A) and similarly inhibited host cell infection by ~60% of control infection (Figure 4.7B). The addition of α6β4 integrin, which based on previously performed α and β integrin-mediated cell adhesion arrays is not expressed on Vero host cells, to the described adherence and infection assays had
Figure 4.6 Specific Anti-integrin Antibodies Inhibit *E. intestinalis* Adherence and Infection. (A) Vero monolayers were pre-incubated with 20 mcg ml⁻¹ anti-integrin antibodies in 2% FBS-supplemented culture medium, or culture medium alone prior to addition of *E. intestinalis* spores, washed to remove unbound spores and evaluated for adherent spores. (B) Vero monolayers were pre-incubated with 5 mcg ml⁻¹ anti-integrin antibodies in 2% FBS-supplemented culture medium, or culture medium alone prior to addition of *E. intestinalis* spores, washed to remove unbound spores, further cultured to allow infection and evaluated for infection of host cells. (A-B) Statistically significant differences from controls were determined using the Student’s *t* test and *p* values of <0.05 (*) and <0.0001 (**) were considered statistically significant and are indicated. Error bars represent standard deviation.

no effect on spore adherence to or infection of host cells, as expected (Figures 4.7C and 4.7D). *In vitro* inhibition of *E. intestinalis* adherence and infection of cells by the addition of exogenous α3β1 or α5β1 integrins supports our hypothesis that integrins are involved in microsporidia adherence and infection and specifically implicates these integrins as putative host cell receptors for *E. intestinalis* spores. Additionally, since the
observed experimental inhibition of spore adherence in this study resulted in a correlating reduction in subsequent host cell infection, the data presented herein also support our hypothesis that spore adherence is a critical element in microsporidia host cell infection.

Figure 4.7 Specific Exogenous Recombinant Integrins Inhibit *E. intestinalis* Adherence and Infection. (A) PBS-diluted recombinant integrins (5 mcg ml⁻¹) in culture medium or PBS/culture medium alone was included during addition of spores to Vero cells. The cells were washed to remove unbound spores and evaluated for adherent spores. (B) PBS-diluted recombinant integrins (5 mcg ml⁻¹) in culture medium or PBS/culture medium alone was included during addition of spores to Vero cells. The cells were washed to remove unbound spores, returned to culture to allow subsequent infection and evaluated for infected host cells. (C) PBS-diluted recombinant integrin (5 mcg ml⁻¹) in culture medium or PBS/culture medium alone was included during addition of spores to Vero cells. The cells were washed to remove unbound spores and evaluated for adherent spores. (D) PBS-diluted recombinant integrin (5 mcg ml⁻¹) in culture medium or PBS/culture medium alone was included during addition of spores to Vero cells. The cells were washed to remove unbound spores, incubated to allow subsequent infection of host cells and evaluated for host cell infection. (A-D) Statistically significant differences from controls were determined using the Student’s *t* test and *p* values of <0.05 (*) and <0.0001 (**) were considered statistically significant and are indicated. Error bars represent standard deviation.

**Discussion**

Data support our hypothesis that host cell integrins play a role in microsporidia adherence and infection *in vitro*. Integrin ligands, including the ECM proteins collagen, laminin, fibronectin and vitronectin significantly inhibited spore adherence and infection in assays of *Encephalitozoon intestinalis* adherence to and infection of Vero (primate)
host cells. These integrin ligands appear to act redundantly via a common mechanism, because pre-incubation of host cells with a combination of these four proteins, compared to pre-incubation with each single protein, resulted in approximately equivalent inhibition of adherence and infection, as opposed to causing an additive or synergistic effect, which would suggest non-redundant activity via discrete individual mechanisms.

Because all of the ECM protein ligands used in this study share the canonical integrin binding amino acid sequence arginine-glycine-aspartic acid (RGD) [18], as do over 50 putative *E. intestinalis* proteins, we evaluated an RDG peptide as a potential inhibitor of spore adherence and infection. Pre-incubation of cells with the RGD peptide alone was sufficient to inhibit spore adherence and infection of host cells. It is not clear from these assays if the ECM proteins and RGD peptide inhibit *E. intestinalis* adherence to host cells by binding integrins and preventing subsequent spore/integrin interactions, or if they operate to inhibit spore adherence via a different, unknown mechanism. It appears possible that the observed inhibition of *E. intestinalis* adherence and infection may be RGD-dependent because each RGD-containing protein and the RGD-peptide exert a very similar effect on adherence/infection.

A previous study indicated that host cell sulfated glycosaminoglycans (GAGs) are involved in microsporidia adherence to host cells [11]. Two experiments were conducted to determine if sulfated GAG-dependent inhibition of *E. intestinalis* adherence and infection and ECM protein-dependent inhibition of adherence/infection might act via a common mechanism. First, Vero host cells were pre-incubated with ECM proteins, exposed to exogenous chondroitin sulfate A (CSA; a sulfated GAG) during spore adherence, or pre-incubated with ECM proteins and exposed to CSA during spore adherence. Both individual treatments inhibited adherence and infection, with CSA resulting in a higher degree of inhibition of adherence (~60% of control, compared to ~40% of control for ECM proteins). Co-treatment with both ECM proteins
and CSA, however, failed to result in any further inhibition of adherence beyond that seen for CSA treatment alone. This suggests that sulfated GAGs and ECM proteins may act via a shared mechanism to inhibit *E. intestinalis* adherence and infection.

Second, to determine if sulfated GAGs are required for ECM protein-dependent inhibition of adherence, *E. intestinalis* adherence assays were carried out in three cell lines concomitantly. CHO-KI cells (adherent cells of hamster origin) have normal expression of host cell sulfated GAGs, while CHO PgsD-677 cells and CHO PgsA-745 cells are deficient in heparan sulfate synthesis and all GAG synthesis, respectively [28,29]. ECM protein or RGD peptide pre-incubation of CHO-K1 cells both significantly inhibited spore adherence to host cells, but pre-incubation with ECM proteins or RGD peptide had no effect on spore adherence to CHO PgsD-677 cells or CHO PgsA-745 cells. This indicates that ECM protein- /RGD peptide-dependent inhibition of *E. intestinalis* is sulfated GAG-dependent, at least in CHO cells; and thus both GAG-dependent and ECM protein- /RGD peptide-dependent inhibition of spore adherence likely operate via a shared, complex mechanism. Heparan sulfate deficiency alone or deficiency in all GAG synthesis both eliminate the effect that ECM proteins or RGD peptide have on *E. intestinalis* adherence to CHO cells, suggesting that ECM protein- /RGD peptide-dependent inhibition of *E. intestinalis* depends largely on heparan sulfate and not chondroitin sulfate, which is expressed in excess on CHO PgsD-677 cells compared to CHO-K1 cells [29].

Notably, in a previous study, spore adherence to confluent CHO PgsD-677 cells and CHO PgsA-745 cells was similarly and significantly reduced (by ~90%) compared to confluent CHO-K1 cells, suggesting that *E. intestinalis* preferentially binds heparan sulfate compared to chondroitin sulfate [11]. Multiple exogenous sulfated GAGs including CSA, however, significantly inhibited spore adherence to host cells [11], indicating that various sulfated GAGs might act in the same manner to reduce putative spore interaction with host cell surface heparan sulfate. In the present study, which
used slightly sub-confluent cell monolayers (~95% confluent), *E. intestinalis* adherence to CHO PgsD-677 cells and CHO PgsA-745 cells was not significantly reduced compared to CHO-K1 cells (data not shown); likewise, significant reduction in spore adherence to GAG-deficient CHO cells was previously determined to depend on complete cell confluence [11]. This suggests that different cell surface molecules are exposed in confluent compared to sub-confluent *in vitro* host cell monolayers, which may drastically affect spore adherence to these cells. In support of this idea, spores can be microscopically observed preferentially binding to the edges of sub-confluent cells (personal observation).

To more directly examine possible host cell integrin involvement in spore adherence and infection, we employed antibodies to integrin α and β chains as potential inhibitors of *E. intestinalis* adherence to Vero host cells. α3, α5, β1 and β2 antibodies all inhibited spore adherence and subsequent host cell infection; additionally, these integrin chains were determined by α and β integrin-mediated cell adhesion arrays to be expressed on the host cells used in our assays. β2 chains do not interact with α3 or α5 chains to form integrins, and are largely expressed on leukocytes, as opposed to epithelial cells, while β1 chains interact with 12 α chains, including α3 and α5, and are expressed on most cells in the body [18,30,31].

We thus focused on determining if α3β1 or α5β1 integrins might be involved in *E. intestinalis* adherence and infection of host cells. α6β4 integrin was chosen as a control because neither α6 nor β4 integrin chains were expressed on the Vero cells used in our assays, and antibodies to these integrin chains failed to inhibit spore adherence to host cells. When the recombinant human integrins tested were added to spore adherence assays, concomitantly with *E. intestinalis* incubation on Vero host cells, α3β1 and α5β1 integrins both significantly reduced spore adherence and infection to host cells by ~60% of control adherence and infection. α6β4 integrin had no effect on spore adherence or
infection. These data support our hypothesis that host cell integrins play a role in microsporidia adherence to and infection of host cells and suggest that \( \alpha 3 \beta 1 \) and/or \( \alpha 5 \beta 1 \) may be host cell receptors or co-receptors for *E. intestinalis* spores. Since either \( \alpha 3 \beta 1 \) or \( \alpha 5 \beta 1 \) integrin caused the same degree of inhibition of adherence/infection, the \( \beta 1 \) integrin chain might be a more important participant than \( \alpha \) chains in spore/host cell interaction.

\( \alpha 3 \beta 1 \) and \( \alpha 5 \beta 1 \) integrins are biologically relevant to microsporidia infection based on both their broad expression in humans, specifically in the intestinal epithelium [31–38], and on the data we present here that indicate their likely importance in spore adherence and infection of Vero host cells *in vitro*. Additionally, these two integrins, or the \( \beta 1 \) integrin chain specifically, are used directly or indirectly, by a large variety of other viruses, bacteria and parasites for attachment and/or invasion of host cells [36,39–46]. Some microbes such as adenovirus, which contains an RGD sequence, and *Shigella* bacteria can bind directly to host cell integrins (\( \alpha 3 \beta 1 \) and \( \alpha 5 \beta 1 \), respectively) to gain access to host cells [36,47]. Other pathogens, however, such as *Streptococcus* and *Staphylococcus* bacteria which directly interact with fibronectin, or the apicomplexan parasite *Toxoplasma gondii* which directly interacts with laminin, use host cell ECM protein integrin ligands to indirectly interact with integrins on the host cell and achieve entry into the cell [40,41,45]. The human herpes virus HHV-8 interacts with heparan sulfate as a binding receptor but requires interaction with \( \alpha 3 \beta 1 \) integrin to enter the host cell for replication [42]. In an even more complex series of events with multiple host cell moieties, the *Neisseria gonorrhoeae* bacterium interacts with host cell heparan sulfate facilitating adherence to the host cell. The bacterium also interacts with the N-terminal end of the host cell fibronectin molecule which allows indirect interaction with the host cell \( \alpha 5 \beta 1 \) integrin via the fibronectin, resulting in uptake of the bacterium by the
host cell [46]. These examples illustrate the many possible interactions with host cell surface moieties that can facilitate microbial adherence to and infection of host cells.

The $\alpha3\beta1$ and $\alpha5\beta1$ integrins, which significantly inhibited *E. intestinalis* spore adherence and host cell infection when added to adherence assays, can each bind all of the ECM protein integrin ligands evaluated [18,48–52], so the possibility remains that the ECM proteins exerted their inhibition of adherence in an integrin-dependent manner. It is unexpected, however that each ECM protein integrin ligand would inhibit spore adherence to the same degree, regardless of whether multiple, distinct integrins or a single host cell integrin were responsible for the observed reduction in adherence and infection, because integrins show a wide range of ligand specificity and binding preference [18]. $\alpha3\beta1$, for example, preferentially binds laminin independently of the RGD sequence, while $\alpha5\beta1$ preferentially integrin binds fibronectin and other RGD-containing ligands [18,30]. Thus, ECM protein-/RGD-dependent inhibition of *E. intestinalis* adherence to host cells may operate via a mechanism distinct from that of the observed integrin-dependent inhibition of adherence. It is also possible that ECM proteins and the RGD peptide may act via an unknown host cell surface moiety or moieties which interact directly or indirectly with host cell integrin(s), instead of interacting directly with integrins via typical or alternative binding interaction. Based on the data presented in this study, we suggest that *in vitro* *E. intestinalis* spores interact with host cells via a complex, multi-component mechanism that involves host cell sulfated GAGs, RGD-containing ECM protein(s), and $\alpha3\beta1$ and/or $\alpha5\beta1$ integrins, perhaps similar to the *Neisseria gonorrhoeae* mechanism which uses heparan sulfate, fibronectin, and the $\alpha5\beta1$ integrin to gain access to host cells. In this study, because the decrease in spore adherence achieved using the herein described molecules as inhibitors in adherence assays correlated with a subsequent reduction in host cell
infection, spore interaction with some or all of the putative host cell participants we have suggested may facilitate, or even be required for infection to take place.

In summary, the addition of exogenous extracellular matrix protein integrin ligands or recombinant α3β1 or α5β1 integrins to assays of *E. intestinalis* adherence/infection significantly reduced spore adherence and infection of Vero host cells. The data from this study support our hypotheses that integrins play a role in microsporidia adherence and infection and that spore adherence is an important event in the process of microsporidia host cell infection and may be required for infection to occur. Further studies will be carried out to determine which specific host cell and microsporidia moieties and molecular interactions are responsible for the integrin-dependent effects observed in this study. Continued elucidation of the infectious process of microsporidia may help facilitate the development of novel therapies for microsporidiosis.

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References


CHAPTER 5

CONCLUSIONS

Investigating the Molecular Mechanisms of Microsporidia Infection

Microsporidia is associated with life-threatening disease in HIV/AIDS patients, severe or disseminated disease in other immunocompromised groups, and asymptomatic or mild disease in the immunocompetent (Didier 2005; Didier and Weiss 2011). Antiretroviral therapy (ART) has reduced opportunistic infections, including microsporidiosis, in HIV positive individuals. However, in areas without access to ART, microsporidiosis continues to cause substantial morbidity and mortality (Didier and Weiss 2011; Wumba et al. 2012). Treatment options for microsporidiosis are limited, variably effective, and associated with significant toxicity (Didier et al. 2004; Anane and Attouchi 2010). Additionally, microsporidia infection in healthy individuals has not been well studied, and the potential short- and long-term consequences of asymptomatic infection have not been determined. Currently little is known about the molecular mechanisms of microsporidia infection, but this information is essential for the development of new effective and safe therapies for microsporidiosis.

Spores adhere firmly to host cell surfaces in vitro and are not removed by repeated washing. During in vivo infection, spore adherence to host cells may be required to ensure the microsporidia polar tube is sufficiently close to pierce or be taken up by target host cells. Our laboratory has thus focused on determining specific host cell and microsporidia spore surface participants in spore adherence in vitro. Our previous studies have shown that: i) host cell sulfated glycosaminoglycans (GAGs) can be used by microsporidia as receptors for adherence to host cells (Hayman et al. 2005); ii) the microsporidia spore surface protein EnP1 binds host cells and participates in spore adherence to host cells in a heparin binding domain-dependent manner.
(Southern et al. 2007); iii) the divalent cations manganese and magnesium augment spore adherence to host cells, likely by activating a spore constituent (Southern, Jolly, and Hayman 2006); and iv) *in vitro* inhibition of spore adherence decreases subsequent host cell infection, while augmentation of spore adherence increases host cell infection (Hayman et al. 2005; Southern et al. 2006; Southern et al. 2007). These studies demonstrate the importance of microsporidia spore adherence in host cell infection and begin to characterize the host cell and spore determinants of adherence and infection. Complete ablation of spore adherence and infection was not achieved in these studies, however, suggesting that additional spore and/or host cell determinants of adherence remain to be resolved.

The goal of the research presented here is to further characterize host cell and spore participants in microsporidia adherence and infection of host cells *in vitro*. The specific aims of this research are i) to characterize an intracellular microsporidia protein and related antibodies to use as a tool in analyses of microsporidia spore surface proteins, ii) to identify putative microsporidia spore surface proteins that participate in spore adherence to and infection of host cells *in vitro*, and iii) to examine the role of host cell integrins in microsporidia adherence to and infection of host cells *in vitro*.

**Hsp70 is Useful for Evaluation of Potential Spore Surface Proteins**

Our first study was conducted to generate a recombinant protein and antibody specific for an intracellular microsporidia protein to use as a control in the evaluation of putative microsporidia spore surface proteins (Jolly et al. 2010). We examined the heat shock protein 70 (Hsp70)-related proteins from *E. cuniculi*, because they are expressed in the cytosol, endoplasmic reticulum (ER), and mitochondria of a cell (Hartl et al. 1992). We chose a highly expressed Hsp70 family member from *E. cuniculi* (ECU02_0100; ‘C1’), which contains all three Hsp70 protein family signature motifs of conserved family members, an N-terminal signal for translocation into the ER, and a C-terminal targeting
sequence for retention in the ER (Hulo et al. 2008). We cloned and expressed the protein C1 Hsp70 and used the purified proteins for antibody generation in rabbits.

Two-dimensional SDS-PAGE and Western analysis were used to confirm that the purified anti-C1 Hsp70 antibodies were specific for C1 Hsp70, and immunoelectron microscopy demonstrated an exclusively intracellular localization of C1 Hsp70 in E. cuniculi spores. As expected, the anti-C1 Hsp70 antibodies did not inhibit E. cuniculi spore adherence to rabbit kidney (RK-13) host cells, indicating their suitability for use in evaluation of potential microsporidia spore surface proteins. In our second study, neither the anti-C1 Hsp70 antibodies nor recombinant C1 Hsp70 had any effect on E. intestinalis adherence to monkey kidney (Vero) host cells, confirming the usefulness of this protein and antibodies to the protein for the evaluation of spore surface proteins in both E. cuniculi and E. intestinalis spore adherence assays. Additionally, the anti-C1 Hsp70 antibodies and recombinant C1 Hsp70 did not cause detectable negative effects on RK-13 or Vero cells (personal observation).

MsADAM Plays a Species-specific Role in Spore Adherence

Our second study was designed to identify spore surface proteins, in addition to the previously described EnP1, that participate in in vitro spore adherence and infection of host cells. We identified a protein (ECU06_0380) named microsporidia ADAM (MsADAM) with domains similar to those of a disintegrin and metalloprotease (ADAM) family glycoproteins, including a predicted signal peptide sequence, prodomain, metalloprotease domain, disintegrin domain, and a transmembrane domain. These domains suggested localization to the spore surface, possible protease activity, and possible integrin binding activity. We thus characterized the localization of MsADAM within E. cuniculi and E. intestinalis spores and, using the anti-C1 Hsp70 antibodies and recombinant C1 Hsp70 characterized in our first study, evaluated the role of MsADAM in spore adherence and infection of host cells.
Immunoelectron microscopy of RK-13 cells infected \textit{in vitro} with \textit{E. cuniculi} and \textit{E. intestinalis} demonstrated that MsADAM localizes to the plasma membrane, exospore, and polar sac-anchoring disk regions of \textit{E. intestinalis} spores; however, MsADAM localized only to the polar sac-anchoring disk complex of \textit{E. cuniculi} spores and was not found in the plasma membrane or exospore. This difference in MsADAM expression was unexpected because of the substantial MsADAM sequence conservation among the \textit{Encephalitozoon} species. \textit{E. intestinalis}, however, exhibits other distinctions from \textit{E. cuniculi}, including the presence of a parasite-secreted fibrillar material within the parasitophorous vacuole, and the presence of spore wall protein 2 (Cali et al. 1993; Hayman et al. 2001; Xu et al. 2006). Thus, the spore surface expression of MsADAM in \textit{E. intestinalis} further highlights the biological difference between \textit{E. intestinalis} and \textit{E. cuniculi}.

In assays of spore adherence, exogenous recombinant MsADAM protein (rMsADAM) inhibited \textit{E. intestinalis}, but not \textit{E. cuniculi}, adherence to host cells, indicating that MsADAM plays a species-specific role in \textit{E. intestinalis} spore adherence. The distribution of MsADAM within a spore thus may have an effect on microsporidia adherence mechanisms. Spore adherence assays carried out with individual recombinant MsADAM metalloprotease and prodomains indicated that the recombinant metalloprotease domain alone (rMET) is sufficient to inhibit spore adherence to host cells, suggesting that this domain is responsible for the observed MsADAM-dependent inhibition of adherence.

Although no protease activity has been demonstrated for MsADAM, putative protease activity may modulate either spore or host cell proteins that participate in adherence. Because disintegrin domains are known to interact with host cell integrins, we expected that the disintegrin domain of MsADAM might be important in spore adherence (Zhang et al. 1998; Eto et al. 2000). We were unable to independently express the disintegrin domain of MsADAM, however, and could not evaluate its effect.
on spore adherence. The rMsADAM- and rMET-dependent inhibition of *E. intestinalis* adherence resulted in a subsequent reduction in host cell infection as expected, providing further evidence that adherence is an important aspect in the microsporidia infection process.

MsADAM, via metalloprotease activity, may also be important for microsporidia functions other than adherence. ADAM metalloproteases are known to cleave ECM proteins, such as collagen, and a wide variety of immune modulatory molecules such as procytokines, progrowth factors, cytokine and growth factor receptors, ligands for apoptosis, and cell adhesion molecules (Yamamoto et al. 1999). ADAM 17 and ADAM 8, for example, cleave TNFα and the IgE surface receptor CD23, respectively (Fourie et al. 2003). If MsADAM is capable of similar proteolytic activity, the protein may play a role in ECM or immune modulation that is beneficial for microsporidia infection and dissemination. Additionally, putative MsADAM interaction with host cell integrins via the disintegrin domain may affect host cell signaling. Pathogenic *Escherichia coli* interaction with integrin α3β1, for example, initiates downstream signaling that induces cytoskeletal rearrangement that facilitates bacterial invasion of host cells (Eto et al. 2007). Integrin signaling events that involve cytoskeletal modulation could be particularly important for microsporidia, such as *E. intestinalis*, that develop in parasitophorous vacuoles within host cells.

While the data presented in our second study suggest that MsADAM, and specifically the MsADAM metalloprotease domain play a role in *E. intestinalis* adherence to and infection of host cells, we have not yet demonstrated that MsADAM binds to integrins or other host cell surface moieties, and specific proteolytic activity of MsADAM could not be determined. Clarification of host cell or spore binding partners or cleavage sequences for MsADAM may be important for elucidation of the microsporidia adherence and infection process. Site directed mutagenesis of the recombinant MsADAM metalloprotease domain may reveal which amino acids of this domain are
required for exogenous rMsADAM to inhibit *E. intestinalis* adherence to host cells. Collectively, the demonstration of MsADAM and EnP1 participation in spore adherence (Southern et al. 2007) suggest that microsporidia adherence to host cells is a complex process that may comprise multiple, distinct interactions.

**Host Cell Integrins Play a Role in Microsporidia Adherence and Infection**

Our third study examined the role of host cell integrins in microsporidia adherence to and infection of host cells *in vitro*. Analysis of the *E. intestinalis* genome revealed over 50 predicted proteins with the RDG integrin-binding motif and the protein herein named MsADAM that has a disintegrin domain. We thus hypothesized that host cell integrins play a role in microsporidia adherence and infection of host cells. In this study, we demonstrated that i) pretreatment of host cells with exogenous extracellular matrix (ECM) protein integrin ligands; ii) pretreatment of host cells with a peptide containing the RGD integrin-binding sequence; iii) pretreatment of host cells with anti-\( \alpha_3, \alpha_5, \beta_1 \) or \( \beta_2 \) antibodies; or iv) addition of exogenous recombinant \( \alpha_3\beta_1 \) or \( \alpha_5\beta_1 \) integrins to assays of *E. intestinalis* adherence/infection significantly reduced spore adherence and infection of host cells. This supported our hypothesis and implicated \( \alpha_3\beta_1 \) and/or \( \alpha_5\beta_1 \) integrins specifically as putative host cell receptors for *E. intestinalis* spores.

The integrin ligands collagen, laminin, fibronectin, and vitronectin appeared to act via a single mechanism to inhibit adherence in these assays because in combination they elicited an identical effect to that of each individual integrin ligand. Furthermore, the RGD peptide also caused a similar degree of effect to the integrin ligands, indicating that RGD sequences in each of these ligands may be responsible for the observed activity. This was unexpected because it is unlikely that each ligand would interact equivalently with a specific integrin or integrins. We originally expected that one or more ligands would bind a specific integrin or integrins to inhibit the adherence of
spores to host cell integrins; however, the data may indicate that integrin ligand- and RGD-dependent inhibition of adherence and infection involves cell surface moieties other than integrins. Alternatively, the data could indicate that an RGD-specific interaction with the β1 integrin chain is responsible for the integrin ligand- and RGD-dependent inhibition of adherence and infection.

Our data suggested that integrin ligand- and RGD-dependent inhibition of adherence and infection may involve cell surface moieties other than integrins, and microsporidia spores have already been determined to interact with host cell sulfated GAGs (Hayman et al. 2005). Thus we conducted assays to determine if the addition of exogenous sulfated GAGs to spore adherence assays and pretreatment of host cells with integrin ligands or the RGD peptide might be inhibiting spore adherence via a common mechanism. Our lab previously showed that the addition of exogenous chondroitin sulfate A (CSA; a sulfated GAG) to spore adherence assays inhibited *E. intestinalis* adherence and infection (Hayman et al. 2005). In this study we compared spore adherence assays carried out on integrin ligand-pretreated cells, spore adherence assays carried out in the presence of exogenous CSA, and spore adherence assays carried out using cotreatment with both integrin ligands and CSA. Adding exogenous CSA to assays caused a more robust inhibition of adherence than pretreatment with integrins ligands. Cotreatment with both integrin ligands and CSA failed to result in any further inhibition of adherence beyond that demonstrated for CSA treatment alone. This suggested to us that sulfated GAGs and integrin ligands may act via a shared mechanism to inhibit *E. intestinalis* adherence and infection.

Some microbes require an interaction with host cells GAGS for a subsequent interaction with other host cell molecules such as integrins. The human herpes virus HHV-8, for example, interacts with heparan sulfate as a binding receptor but requires interaction with α3β1 integrin to enter the host cell for replication; preincubating the virus with heparan sulfate prevented viral adherence to host cells and subsequent viral entry
into host cells (Wang et al. 2003). To determine if sulfated GAGs are required for integrin ligand-dependent inhibition of adherence, *E. intestinalis* adherence assays were carried out in CHO-KI cells with normal expression of host cell sulfated GAGs, and CHO PgsD-677 cells and CHO PgsA-745 cells that are deficient in heparan sulfate synthesis and all GAG synthesis, respectively (Esko et al. 1985; Esko et al. 1988). ECM protein or RGD peptide pre-incubation of CHO-K1 cells significantly inhibited spore adherence to host cells, but had no effect on spore adherence to CHO PgsD-677 cells or CHO PgsA-745 cells. This indicates that integrin ligand- and RGD peptide-dependent inhibition of *E. intestinalis* requires host cell heparan sulfate but not chondroitin sulfate, which is expressed in excess on CHO PgsD-677 cells compared to CHO-K1 cells (Esko et al. 1988).

**Proposed In Vitro Model of Microsporidia Adherence**

The data presented in these studies collectively provide support for MsADAM and host cell integrin involvement in adherence and infection and further suggests that microsporidia adherence and infection of host cells requires more than one host cell surface component. We propose an *in vitro* model in which microsporidia interacts with host cell surface sulfated GAGs, such as heparan sulfate, α3β1 and/or α5β1 integrins, and endogenous ECM proteins to facilitate adhesion to and infection of host cells (Figure 5.1). To evaluate the plausibility of this model, the ability of spores to directly or indirectly bind α3β1 and/or α5β1 integrins must be determined. This might be accomplished in cells with normal GAG and ECM protein expression, but with manipulated integrin expression, to remove the possibility of modulating other host cell molecules that may be required for putative microsporidia/integrin interaction. Integrin knock-out or knock-down in host cells could facilitate such an evaluation. Additionally, because spore interaction with integrins may be expected to result in downstream integrin signaling, an examination of known integrin signaling cascades in cells exposed
to microsporidia spores, proteins, or peptides may provide evidence of microsporidia interaction with integrins and may help clarify the role integrins play in adherence and infection of host cells. It remains to be seen, for example, if MsADAM might interact with host cell integrins and what implications this would have in the adherence and infection process.

Figure 5.1 Proposed In Vitro Model of Microsporidia Adherence

The data presented in this study also support our hypothesis that spore adherence to host cells is an integral part of subsequent infection of these cells. While microsporidia, because of their ability to “inject” infectious sporoplasm into a host cell via the everted polar tube, do not have the obvious requirement for entry into host cells
that viruses, for example, have; in all cases presented here and in our previous studies reduction of spore adherence to host cells also reduces subsequent infection (Hayman et al. 2005; Southern et al. 2007). Although we have not provided evidence that spore adherence is required for infection, we have showed that augmenting spore adherence also augments spore infection, further supporting the role that adherence and infection are intimately related.

Several lines of evidence demonstrate that while microsporidia adherence may be required for infection, adherence alone is not sufficient to ensure host cell infection. First, UV and chemically inactivated spores still adhere in significant numbers to host cells but cannot cause infection (Southern et al. 2006, personal observation). Second, while manganese and magnesium divalent cations augment both microsporidia adherence and infection, calcium fails to augment adherence yet significantly augments host cell infection (Southern et al. 2006). This may represent an increase in spore germination given the role calcium is thought to play in polaroplast and posterior vacuole swelling during germination (Pleshinger and Weidner 1985; Xu and Weiss 2005). And finally, *E. intestinalis* spores adhere equally well to Vero and CHO-K1 cells but infect CHO-K1 cells at an extremely reduced efficiency compared to Vero infection (personal observation). This indicates that despite the ubiquitous distribution of GAGs and integrins on human cells and the likely role of these molecules in microsporidia adherence to host cell, targets for modulation of microsporidia infection might be manipulated to reduce or eliminate host cell infection. An understanding of the process of microsporidia infection at the molecular level, however, will be required to exploit specific targets that might facilitate the development of new therapies for microsporidiosis.
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VITA

CORY A. LEONARD

Personal Data: Date of Birth: February 20, 1976
Place of Birth: St. Augustine, Florida
Marital Status: Married

Education: B.A. Biology, University of North Carolina at Asheville,
Asheville, North Carolina 1999
Ph.D. Biomedical Sciences, East Tennessee State
University, Johnson City, Tennessee 2013

Professional Experience: Laboratory Technologist, Microbiology Department,
Genova Diagnostics, Asheville, NC 1999-2007

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Viral Immune Response. Current Immunology
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Honors and Awards: ETSU School of Graduate Studies, Graduate Student
Research Grant, 2011
Appalachian Student Research Forum, Second Place Poster
Presentation Award, 2012