INVESTIGATION OF THE MOLECULAR AND MECHANISTIC BASIS FOR ATTACHMENT BY GIALDIA LAMBLIA

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ABSTRACT

Giardia lamblia is a protozoan parasite responsible for widespread diarrheal disease in humans and animals. Attachment to the host intestinal mucosa is necessary for establishing infection, but how Giardia performs this attachment is poorly understood. This work illuminates two aspects of Giardia attachment: a molecular analysis to identify microfilament-associated proteins and a mechanistic analysis to examine the dynamics of parasite attachment and detachment.

The microfilament system plays an important role in attachment, yet no known microfilament protein except actin in Giardia has been identified. We developed an actin co-sedimentation/mass spectrometry assay using human β-actin to identify possible microfilament-associated proteins in Giardia. We found that only Giardia actin and two proteins in the α-giardin family (α-1 giardin and α-7.3 giardin) were specifically enriched in F-actin pellets (P<0.2). The specific co-existing of Giardia actin and F-actin suggests that the parasite and human actins can co-polymerize, an interesting finding for such divergent species. The two α giardins identified in this study belong to annexin family and localized to the plasma membrane, where actin also localizes, supporting an in vivo role for the observed in vitro interactions.
The dynamic changes of the relative topology of substrate and parasite have never been observed and would provide important evidence to understand the attachment mechanism. We used total internal reflection fluorescence (TIRF) microscopy to observe parasites surface-labeled with Alexa-488 conjugated wheat germ agglutinin attach to a glass substrate. We found that the bare zone (a cytoplasmic protrusion through the structurally-unsupported center of the ventral disk), the lateral crest at the periphery of the ventral disk, and the lateral shields at the posterior end of the ventral disk are in closest apposition to the substrate, with the bare zone showing most the dramatic changes during attachment and detachment. In addition, observations of the dynamics of fluorescent microspheres indicated the presence of fluid flow under the surface of the ventral disk of attached parasites. These data provide support for a negative pressure model of attachment.

Together these studies advance our understanding of both the machinery of attachment and the biomechanical properties of attachment and point to new directions for chemotherapeutic research.
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TABLE OF CONTENTS

CHAPTER 1………………………………………………………………………….1

Introduction

1.1 Importance of *Giardia* research……………………………………………….2

1.1.1 History………………………………………………………………………….2

1.1.2 Clinical and economic relevance………………………………………….2

1.2 *Giardia* biology………………………………………………………………….5

1.2.1 Structures……………………………………………………………………….5

1.2.2 Two nuclei………………………………………………………………………6

1.2.3 Life cycle…………………………………………………………………………7

1.2.4 Metabolism……………………………………………………………………..8

1.2.5 Evolution…………………………………………………………………………9

1.3 *Giardia* pathology………………………………………………………………10

1.3.1 Pathology……………………………………………………………………….10

1.3.2 The role of attachment in *Giardia* pathology……………………………11

1.3.3 Current treatments…………………………………………………………….12
1.4  *Giardia* cytoskeleton.................................................................13
1.4.1 Eukaryotic cytoskeleton...............................................................13
1.4.2 The richness of the *Giardia* microtubule system..............................15
1.4.3 Incomplete microfilament system in *Giardia*.......................................17
1.5 Proposed mechanism of attachment...................................................19
1.5.1 Previous research on attachment................................................19
1.5.2 The adhesion model of attachment.................................................21
1.5.3 The grasping model of attachment...............................................22
1.5.4 The hydrodynamic model of attachment.........................................23
1.5.5 New observations...........................................................................23
1.6 Drug studies of attachment ..............................................................24
1.6.1 Summary of previous studies.......................................................24
1.6.2 Our recent research........................................................................25
1.7 Summary of previous microscopy observations of *Giardia*...................26
1.7.1 Plasma membrane observations by microscopy ...............................26
1.7.2 Microscopy observation contributions to secretory pathway studies........27
1.7.3 The reproduction pattern observed by microscopy............................29
1.7.4 Microscopy observations for *Giardia* attachment .................................29

1.7.5 Microscopy observations for *Giardia* flagella ..................................31

1.8 Proposed tools and methodology .............................................................32

1.8.1 Co-sedimentation method .................................................................32

1.8.2 Total internal reflection fluorescence microscopy method ....................33

1.9 Figures .....................................................................................................36

CHAPTER 2.....................................................................................................38

Identification of microfilament-associated proteins in *Giardia lamblia*

2.1 Abstract ..................................................................................................39

2.2 Introduction ............................................................................................40

2.3 Materials and methods ...........................................................................43

2.4 Results ....................................................................................................50

2.5 Discussion .............................................................................................56

2.6 Figures and tables ..................................................................................62

CHAPTER 3.....................................................................................................71
Analysis of *Giardia* attachment by TIRF microscopy support a negative-pressure model of attachment

3.1 Abstract........................................................................................................72

3.2 Introduction.................................................................................................73

3.3 Materials and methods..............................................................................77

3.4 Results.........................................................................................................80

3.5 Discussion...................................................................................................89

3.6 Figures and tables.......................................................................................95

CHAPTER 4......................................................................................................106

Conclusion and discussion

4.1 Conclusion..................................................................................................107

4.2 Discussion.................................................................................................107

4.2.1 Identification of \( \alpha \)-1 and \( \alpha \)-7.3 giardins as microfilament-associated proteins..............................................................................................................................107

4.2.2 TIRFM observation and analysis of *Giardia* attachment......................110

4.3 Future directions.........................................................................................112

4.3.1 Protein research.......................................................................................112
CHAPTER 1

Introduction
1.1 Importance of *Giardia* Research

1.1.1 *History*

*Giardia* was first described by Antonin van Leeuwenhoek in 1681. Leeuwenhoek examined his own diarrheal stool by microscopy and named an organism he observed in it “animacule” (Dobell, 1920). Almost two centuries later, this organism was more fully described by physician Vilém Dušan Lambl in 1859 (Lambl, 1859). Lambl named this organism *Cercomonas intestinalis* because he thought it belong to the genus *Cercomonas*, then in 1888 zoologist Raphael Anatole Émile Blanchard changed it to *Lamblia intestinalis* in honor of Lambl’s contribution (Blanchard, 1888). At the same point in history, Johann Künstler observed a related organism in tadpoles that he called *Giardia*, the first time *Giardia* was designated as a genus name, chosen to honour Alfred Mathieu Giard’s detailed description of the organism. Finally, *Giardia lamblia* was first given as a name for the human parasite in 1915 in Kofoid and Christiansen’s publication (Kofoid and Christensen, 1915). Since then other names have been used to describe this organism, such as *G. enterica* (Kofoid, 1920), *G. duodenalis* (Stiles, 1902), and *G. intestinalis* (Kulda and Nohynkova, 1996). Nowadays, *G. lamblia*, *G. duodenalis* and *G. intestinalis* are the three most commonly used names. Because investigators delineate *Giardia* species either by the host species or by morphology characteristics, the number of *Giardia* species remains controversial. Ascribing to the convention of the genome project, we will use *Giardia lamblia* throughout this dissertation to refer to all strains of the parasite that infect humans.

1.1.2 *Clinical and economic relevance*
At first, *Giardia* was not recognized as a pathogen. Although Giard described the presence of *Giardia* in stools of children with diarrhea, he did not connect this protozoan to the cause of diarrhea. It was not until the mid-twentieth century that Rendtorff first described *Giardia* as a pathogen of human and animals (Rendtorff, 1954). Today *Giardia lamblia* is recognized as one of the most prevalent intestinal parasites and cause of a diarrheal disease called giardiasis throughout the world (Wolfe, 1992). The NIH lists *Giardia* as a Category B Priority Pathogen (NIAID Category A, B & C Priority Pathogens).

Patients with giardiasis are most often asymptomatic. When symptoms develop, they do so within two weeks of infection (Ortega and Adam, 1997). The most common symptoms include gastrointestinal disorders, malabsorption, weight loss, and fatigue lasting for two or more weeks. Both asymptomatic carriers and symptomatic patients can transmit the cyst stage of the parasite to other individuals through fecal-oral contact. *Giardia* infections in some individuals, especially those who are immunocompromised, can have a severe impact on health (Lengerich et al., 1994). Additionally, people with chronic *Giardia* infections, particularly children, are at risk for long-term growth retardation and delayed cognitive development (Fraser et al., 2000; Berkman et al., 2002; Niehaus et al., 2002; and Ricci et al., 2006). The reason for the wide spectrum of symptoms and severity of infection is not fully understood. It may be due to either the difference between strains or genotypes, or difference of host genotype or immune response (McIntyre et al., 1986).

Outbreaks of giardiasis have influenced human behavior throughout history. For
example, *Giardia* was identified as one cause of the dysentery afflicting crusaders in Palestine in the 12th and 13th centuries (Mitchell et al, 2008). Recently, giardiasis outbreaks have also happened in developed countries. In 1998, a *Giardia* outbreak was reported in Sydney, Australia, and there was also an outbreak in Bergen, Norway in 2004.

Because *Giardia* is transmitted via food and water, and is partially resistant to usual environmental stresses and sewage treatments, like chlorine and UV, it is a major contributor to diarrheal disease worldwide. This is especially true in developing countries where the insufficiency of clean water means that *Giardia* infections are endemic (Leclerc et al., 2002). The World Health Organization (WHO) estimates that more than 3.5 billion people are at risk for *Giardia* infections, while 1 billion people are infected annually (WHO, 2008). The long-term health risks associated with chronic *Giardia* infections of growth retardation and delayed cognitive development pose significant public health challenges for a country (Fraser et al, 2000; Berkman et al., 2002; Niehaus et al., 2002; and Ricci et al., 2006). Undoubtedly this situation increases the economic burden for the people in developing countries that are already suffering from insufficient food supplies and many endemic infectious diseases. Even in the U.S., *Giardia* is a primary cause of waterborne outbreaks of diarrhea (Barwick et al., 2000). The annual incidence of giardiasis in the U.S. is primarily in campers or children in daycare centers. The reported number is ~20,000 cases annually, while the actual number is thought to fluctuate between 2.0 and 2.4 million annually (Furness et al., 2000 and Hlavsa et al., 2005).
In addition to human infections, *Giardia lamblia* is also capable of infecting a wide range of animal hosts including domesticated cats and dogs, cattle, and other livestock. These veterinary infections have inevitably increased agricultural expenses. Obviously giardiasis is a worldwide health problem. The full understanding of *Giardia* pathology and biology is crucial to help us develop tools to fight against giardiasis.

1.2 *Giardia* Biology

1.2.1 Structures

*Giardia lamblia* is a unicellular organism measuring approximately 12 μm in length and 8 μm in width. The parasite has a flat polarized morphology. The most notable aspects of *Giardia* trophozoites are two cytoskeleton structures that are unique to the genus.

The first is the elaborate and unique domed structure called the ventral disk, which is just under the ventral plasma membrane. The concavity of the ventral disk increases upon attachment (Sousa et al., 2001). The ventral disk is composed of spiral arrays of microtubules from which dorsal ribbon structures extend into the cytoplasm (Holberton, 1973a and 1981; and Mueller et al., 1974). When viewed by transmission electron microscopy observation, the ribbons have a striated appearance (Feely et al., 1984). The absence of microtubules at the center of disk define a small area termed the “bare zone”, while at the periphery of the disk, a network of fibers of unknown composition called the lateral crest replaces microtubules and ribbon structures (Holberton, 1973a, b). The ventrolateral flange is a flexible overhang of the cell body around the anterior edge of disk. Interestingly, the ventrolateral flange and lateral crest
have been observed to interact with epithelial cells, suggesting a role during attachment (Friend, 1966; Erlandsen, Feely, 1984; Sousa et al., 2001).

There are also eight flagella grouped into four pairs termed anterior, posterior-lateral, caudal, and ventral flagella, respectively. The flagella have a typical eukaryotic 9+2 arrangement of microtubules that emerge from basal bodies. The pairs of flagella are rooted deeply and together in the cell body slightly anterior to the two nuclei. At the posterior end of the ventral disk, there is a notch and the ventral flagella emerge posterior to here. On the posterior side of the anterior and posterior lateral flagella ‘dense rods’ structures appear as electron dense matter (Filice, 1952). Additionally, there are ‘striated fibers’ or ‘marginal plates’ running along the anterior axonemes and extending to the ventrolateral flange (Holberton, 1973a; Sogayar, Gregorio, 1991). Some short arrays of microtubules, termed the ‘funis’ are associated with the caudal flagella on the dorsal and ventral sides (Kulda, Nohynkova, 1978).

In the middle of cell body, there is a poorly defined microtubule structure called the median body, which looks like a disordered assemblage of microtubules. The median body is present in all *Giardia* species and appears to change its appearance during the cell cycle (Elmendorf et al., 2003). The function of median body is not clear but the morphology of median body can be used as an index for distinguishing different species.

1.2.2 *Two nuclei*

*Giardia* trophozoites have two nuclei. A strain with a single nucleus has never been observed, suggesting that maintenance of two nuclei is required. The two nuclei are located in the anterior part of cell body and are symmetric along the long axis. Their
appearance is the same as observed by electron microscopy observation (Gillin et al. 1996; Adam, 2001), and studies using flow cytometry analysis show that the two nuclei contain equal amounts of DNA (Bernander et al. 2001). Recently nucleoli were identified (Jiménez-García et al., 2008) and further visualized by transmission electron microscopy and laser scanning confocal microscopy (Tian et al., 2010). Each of the two nuclei contains a single granular nucleolus. Studies have also shown that both nuclei replicate at approximately the same time (Wiesehahn, Jarroll et al. 1984) and both are transcriptionally active (Kabnick and Peattie, 1990). These qualities distinguish Giardia from the ciliated protozoa that contain macro and micronuclei with distinct morphologies and functions (reviewed by Adam, 2001).

1.2.3 Life cycle

Giardia has two distinct life stages: environmentally-resistant cysts and vegetative trophozoites (Gillin et al., 1996; Lujan et al., 1997). The cyst is metabolically quiescent, allowing longer periods of survival in nature and remains infective for up to 16 days in the environment (Rendtorff and Holt, 1954). Cysts are partially resistant to the standard chlorine concentrations used in most water purification systems (Jarroll et al., 1981a). Initiation of infection can begin by ingestion of as few as 10 cysts via contaminated water or food consumed by host (Wolfe, 1992; Farthing, 1997). In the digestive tract of the host, a process called excystation results in the transformation from cyst to vegetative trophozoite form. During the trophozoite stage, the parasites attach to the epithelial cells of the small intestine to prevent premature excretion by peristalsis and replicate to generate more trophozoites to colonize the intestinal surface. Eventually,
some trophozoites in the jejunum are triggered by the altered luminal conditions, including alkaline pH of 7.8, conjugated bile salts and fatty acids (Gillin et al., 1988), to undergo encystation, and finally cysts are excreted, waiting for the next host to infect.

For a long time *Giardia* was thought to reproduce only asexually. Three lines of more recent evidence, however, argue for the presence of a sexual cycle, although the sexual stages remain undefined: (1) Recent research based on sequencing analysis revealed evidence of meiotic recombination between parasite populations in various human hosts in a small village (Ramesh et al., 2005); (2) Putative homologues of several important genes involved in meiotic recombination have been identified (Poxleitner et al., 2008; Logsdon, 2008); (3) *Giardia* has very low levels of allelic sequence heterozygosity between *Giardia* strains or assemblages, while a long asexual reproduction history should have resulted in high levels of nucleotide substitutions (Teodorovic et al., 2007).

1.2.4 Metabolism

*Giardia* depends primarily on anaerobic metabolism. The products of glucose metabolism are acetate, ethanol, alanine, and carbon dioxide. Which is the main product depends on subtle changes in the oxygen concentration in the environment. For example, under strict anaerobic condition, alanine is the product (Edwards et al., 1989; Paget et al., 1990 and 1993). Interestingly, in *Giardia* pyruvate-ferredoxin oxidoreductase (PFOR) catalyses the conversion from pyruvate to acetyl coenzyme A, while most eukaryotes utilize pyruvate dehydrogenase complex for this purpose (Lindmark, 1980; Townson et al., 1994 and 1996).

Amino acids are another important energy source for *Giardia*. One well recognized
amino acid metabolism pathway is the arginine dihydrolase pathway (Edwards et al., 1992; Schofield et al., 1990 and 1992). Correspondingly, two enzymes called arginine deiminase and ornithine carbamoyltransferase participating in this pathway are very abundant proteins in *Giardia*. This pathway is common in prokaryotes but rare in eukaryotes, which may reflect either the evolutionary history of *Giardia* or the pressures of its niche within the anaerobic intestinal environment.

As a parasite, *Giardia* can rely on the host’s resources for its requirements for amino acids, lipids, purine and pyrimidine (reviewed by Adam, 2001). It is therefore not surprising to know *Giardia* trophozoites cannot synthesize most fatty acids (Jarroll et al., 1981b; Ellis et al., 1996) and depends on salvage pathways for both purine and pyrimidine nucleosides (Wang and Aldritt, 1983; Aldritt et al., 1985).

### 1.2.5 Evolution

*Giardia* belongs to the order Diplomonadida and the family Hexamitidae. An interesting characteristic of *Giardia* is the absence of some classic eukaryotic cellular features, including mitochondria and peroxisomes. It also has a diminished Golgi complex prior to encystation (Tibayrenc et al., 1990; Lujan et al., 1995; Gillin et al., 1996). But the discovery of genes of mitochondrial origin in *Giardia* (Roger et al., 1998) indicates that *Giardia* diverged after the origin of mitochondria and later experienced a secondary loss of the organelle. The recent identification of mitosomes in *Giardia* seems to provide evidence for a remnant of this organelle (Tovar et al., 2003; Regoes et al., 2005).

Generally the evolutionary position of *Giardia* is determined by 16S rRNA
sequences. Based on the comparison, *Giardia* was proposed as an early branching eukaryote (Boothroyd, et al., 1987; Edlind and Chakraborty, 1987; Sogin et al., 1989). Other genes involving translation and transcription mechanisms also support the basal position of *Giadina* in eukaryotic organism (Hashimoto et al., 1994 and 1995). The comparisons based on cytoskeleton genes are not in agreement. The actin gene shows an early divergence status, while tubulin genes suggest a more recent divergence (Edlind et al., 1996). Generally, most research places *Giardia* as an early diverging eukaryote, although a minority of phylogenetic analyses provide contradictory evidence for a more recent divergence and stronger selective pressures. The current placement of *Giardia* evolution awaits further resolution.

### 1.3 *Giardia* Pathology

#### 1.3.1 Pathology

Unlike other microbes that directly penetrate the epithelial barrier, invade surrounding cells and tissues, or enter the bloodstream of host, *Giardia* trophozoites attach to intestinal epithelial cell surface and remain in the lumen of the small intestine. This attachment behavior is important to permit the parasite to remain in the intestine for long enough to replicate and results in intestinal damage. People infected with giardiasis can have different disease symptoms ranging from an asymptomatic state to severe clinical presentations including vomiting, diarrhea and weight loss. However, it is still not clear how infections with *Giardia* result in these symptoms. Some possible explanations have been proposed to explain the relationship between parasite and clinical symptoms: direct damage to the microvilli of small intestinal epithelial cells as a
consequence of attachment (Yardley et al., 1964; Hartong et al., 1979; Buret et al., 1990, 1991; Buret, 2007; Koudela, 1994; Katelaris et al., 1995; Chávez et al., 1995; Sousa et al., 2001); the host immune response directed at the parasite (MacDonald and Spencer, 1988; Eckmann 2003; Scott et al., 2000 and 2004; Buret, 2005); toxins released from parasites (Chávez et al., 1995; Djamiatum and Faubert, 1998); reduced absorptive surface of intestinal mucosa due to colonized parasites (Farthing et al., 1997); and interruption of normal function of digestive tract by reducing disaccharidase activity, hypersecretion of chloride, and malabsorption of glucose, sodium, and water (Anand et al., 1985; Khanna et al., 1988; Belosevic et al., 1989; Buret et al., 1991, 1992 and 2002; Cevallos et al., 1995; Dagci et al., 2002; Chin et al., 2002; Troeger et al., 2007).

1.3.2 The role of attachment in Giardia pathology

Although the mechanism of Giardia trophozoite attachment is controversial, it is well established that their ventral sides contact the substrate surface when attached. Early scanning electron microscopy (SEM) studies based on the observation of rat and human intestinal cells infected with Giardia by Erlandsen and Chase demonstrated that the surface of epithelial cells was covered with numerous parasites during infections and some circular shaped lesions on epithelial surface were presumably caused by once attached and now detached Giardia (Erlandsen and Chase, 1974). The shapes and diameters of these lesions correspond to a structure on the ventral side of Giardia called the ventral disk. In the same study, transmission electron microscopy (TEM) data revealed the microvilli within the disk shaped lesion were displaced and deformed. Furthermore the length of the microvilli was decreased in the lesion area. Thus,
Erlandsen and Chase suggested that the combination of mechanical blockage of the mucosal surface and the direct injury to the microvillus border, a result of parasite attachment, was likely responsible for the pathology associated with giardiasis. A study in 1990 also revealed the correlation between the progression of giardiasis and both the number of *Giardia* attached to and the damage to the host intestinal surface (Khanna et al., 1990). No matter what the exact mechanism is for the pathology of *Giardia*, the attachment of *Giardia* trophozoites to host small intestine epithelial cells is required for reproduction in a suitable environment and establishment of infection. Thus treating giardiasis by drugs blocking attachment but not interrupting the normal function of host cells is a potential direction to treat *Giardia* infection.

1.3.3 **Current treatments**

Although there is no effective vaccine for humans to prevent *Giardia* infection, treatment of giardiasis is possible with drugs that disrupt the *Giardia* metabolic pathways including metronidazole, tinidazole, ornidazole and secnidazole, and alternative drugs like quinacrine, furazolidone and albendazole, are used to treat giardiasis (Gardner and Hill, 2001). In the U.S. the most common treatment for giardiasis is metronidazole, which treats giardiasis through conversion into a toxic nitro radical product in parasites by PFOR of *Giardia*. But its treatment is typically less than 90% effective, and the reoccurrence rates of giardiasis are as high as 90% (Zaat et al., 1997; Zaat et al., 2000). Also, drug resistance rates of up to 20% have been reported (Boreham et al., 1988; Farthing, 1997). Other drugs are less than ideal due to toxicity and side effects (Zaat et
al., 1997; Gardner and Hill, 2001). Thus, the development of new drugs to treat giardiasis is an important area for future research.

1.4. **Giardia Cytoskeleton**

1.4.1 *Eukaryotic cytoskeleton*

The cytoskeleton is one of the hallmark features of eukaryotic cells. The classical cytoskeleton system can best be divided into four basic components: (1) microfilaments; comprised of actin and associated proteins; (2) microtubules; comprised of tubulins and associated proteins; (3) intermediate filaments and associated proteins; and (4) motor proteins associated with microfilaments or microtubules. Although different components may have distinct evolutionary pathways or origins, they work together to provide architectural support and to perform dynamic functions.

Microfilaments are helical 5-9 nm diameter fibers formed from polymerized actin monomers. Free, globular actin monomers are referred to as G-actin, while the polymerized form found in microfilaments is referred to as F-actin. Actin is the most abundant protein in the typical eukaryotic cell, achieving concentrations greater than 100 µM (Pollard et al., 1999). Actin shares an evolutionary history with a bacterial homologue, MreB (Rubenstein and Wen, 2005). Chicken and human skeletal muscle actin are 100% identical, and even the actin proteins of yeast and humans still share more than 80% sequence identity. Higher eukaryotes such as mammals have different actins divided into three classes: α-, β-, and γ-actins. α-actin is the major component of muscle, while β- and γ-actins can be found in non-muscle cells.

Microfilaments participate in determining cell shape; enabling cell movements by
lamellipodia, filopodia, or pseudopodia; and cell division during cytokinesis by a contractile ring (Feierbach and Chang, 2001). More than 60 distinct protein classes of actin-binding and microfilament-associated proteins have been found (Pollard and Borisy, 2003). Actin binding proteins can be divided into several groups, including: monomer binding proteins (thymosins and profilin), small severing proteins (cofilin), capping proteins (capZ and gelsolin), cross-linking proteins (Arp2/3 complex, α-actinin and formin), side-binding proteins (coronin), and motor proteins (myosins) (Pollard, 1990 and 1999; Puius et al., 1998; McGough, 1998). Myosins, a large family of proteins with up to 16 different classes, are the only identified actin motor protein.

Microtubules are 25 nm cylinder structures formed by polymerized heterodimers of α- and β-tubulin (Kirschner, 1978). Tubulin was traditionally thought to be specific to eukaryotes but in prokaryotes, FtsZ was shown to be evolutionarily related to tubulin (Nogalas et al., 1998). Microtubules have three essential roles in eukaryotes: first, microtubules are the predominant component of eukaryotic cilia and flagella used for cell motility; second, microtubules are the major component of the mitotic spindles used for cell division; third, microtubules are important structures in the cytoplasm used for tracks for vesicular trafficking. Microtubules in vivo are highly dynamic and assemble/disassemble as regulated by microtubule associated proteins (MAPs); the exact mechanisms and functions of these MAPs are complex and vary significantly among different eukaryotes. The two classes of motor proteins associated with microtubules are kinesins and dyneins. Different kinesins and dyneins have specific functions such as intracellular transport, flagellar motility, and spindle fiber dynamics (reviewed by
Intermediate filaments are helical structures formed from polymers of intermediate protein monomers; their size is intermediate between microfilaments and microtubules, hence the derivation of their name. They usually consist of one, two or three different intermediate proteins and contribute to cell structure and strength. In total, the intermediate protein family is composed of more than 50 proteins (Goldman and Chao, 1999). Recent work revealed that there are also intermediate filament associated proteins. However, relatively less research has focused on intermediate filaments compared to microfilaments and microtubules. In protists, the existence of intermediate filament proteins beyond the nuclear lamins remains a subject of debate (Erber et al., 1998).

1.4.2 The richness of the Giardia microtubule system

Based on the conservation of the cytoskeleton system across the eukaryotic domain, we would expect the typical cytoskeleton system composition to be present in Giardia as well. Molecular research has shown that the microtubule system is relatively complete. However only actin itself has been found in Giardia, but none of the other microfilament associated proteins suggesting that proteins novel to Giardia form the other members of microfilament system in Giardia.

In the microtubule system, the proteins that have been identified include: α-, β-, γ-, δ- and ε-tubulin (Holberton et al., 1981; Kirk-Mason et al., 1988; Nohira et al., 1992; Nohynkova et al., 2000, McArthur et al., 2000 and Morrison et al., 2007), centrin (Belhadri, 1995 and Meng et al., 1996a), a new group of Giardia specific MAPs: β-
giardin and δ-giardin (Nohira et al., 1992; Weber et al., 1993), and several larger coiled-coil proteins whose functions are not well described (Marshall and Holberton, 1993, 1995; Elmendorf et al., 2003). In 2006 Richardson et al. identified 24 kinesins in *Giardia* and they belong to 10 of 14 kinesin families (Richardson et al., 2006). A similarity search of the *Giardia* genome database indicates 12 dynein-related sequences (Morrison et al., 2007 and reviewed in Elmendorf et al., 2010). In addition, ultrastructure evidence shows that dynein-like arms protrude from microtubule structures in *Giardia*, including the flagella axonemes, the median body, and the funis (Campanati et al., 2002).

Tubulins are mainly distributed in interphase cells in the ventral disk, flagella and median body. Among them, α-tubulin and β-tubulin are the major components of the ventral disk and flagella, while γ-tubulin localizes to the basal bodies (Nohynkova et al., 2000). Centrin is a calcium binding protein that has an essential role in the duplication of the centrosomes during the cell cycle and in microtubule severing (Salisbury, 1995); research localized centrin to a subset of microtubule structures in *Giardia* including the basal bodies, all the flagella axonemes, the ventral disk, funis, and the median body by immunofluorescence (Meng et al., 1996a; Correa et al., 2004). For other *Giardia* microtubule-associated proteins including β-giardin and δ-giardin, our knowledge of them concentrates on their localization rather than their precise functions: the β-giardin assembles into 2.5 nm filaments that further assemble into the superstructures of the dorsal ribbons of the ventral disk (Holberton, 1981; Crossley and Holberton, 1985). Likewise δ-giardin localizes to the ventral disk (Jenkins et al., 2009).

Importantly, α–giardins, an annexin-like protein family, incorrectly share the
name giardin with β-giardin and δ-giardin (Weiland et al., 2005). The α-giardins appear to share neither sequence nor structure similarity with β-giardin and δ-giardin. The large family of α–giardins (at least 17 members) have a variety of localizations in the cell, including the dorsal plasma membrane, the flagella and the adhesive disc (Wenman et al., 1993; Weiland et al., 2005). These locations suggest α–giardins may be components of cytoskeleton system.

1.4.3 Incomplete microfilament system in Giardia

In 1982 Feely et al. claimed to have identified α–actinin, tropomyosin, and actin motor protein myosin in Giardia by indirect immunofluorescence assay using heterologous antibodies (Feely et al., 1982). Later, Giardia actin was identified as well (Drouin et al., 1995). But genome evidence indicates the actin associated proteins identified by Feely et al. are not present in the Giardia genome (McArthur et al., 2000; Elmendorf et al., 2003), suggesting the immunofluorescence data was likely an artifact of cross-reacting antibodies.

Our lab also searched the Giardia genome for actin-binding and microfilament-associated proteins using a bioinformatics approach (Morrison et al., 2007; Elmendorf et al., 2010). This is interesting because the possible candidates regulating microfilament assembly/disassembly dynamics and organization are typically well-conserved. Sequences from a broad diversity of other eukaryotes, including S. cerevisiae, D. discoidium, D. melanogaster and H. sapiens, were chosen as probes for searching the Giardia genome by BLAST. Supplemental methods such as PSI-BLAST searches and Hidden Markov Model (HMM) searches were also performed.
These studies verified that the *Giardia* genome contains only one actin gene. The *Giardia* actin has higher homology to eukaryotic non-muscle (β) actins than to muscle (α) actin, and the comparison between *Giardia* actin and human β-actin reveals 60.5% identity and 79.2% similarity at the amino acid level (Figure 1.1), which is evenly distributed across the entire sequence. Although the genes of numerous microtubule associated proteins are highly conserved and can be readily identified in the *Giardia* genome, we cannot find homologous sequences to any common actin/microfilament associated proteins (Morrison et al., 2007; Elmendorf et al., 2010). This is a surprising result that is likely not due to an inefficiency of the BLAST method because we can find a homolog of yeast Sec23 which contains a gelsolin repeat, and we can easily identify three actin related protein (Arp) genes, although all other members of the traditional Arp1 and Arp2/3 complexes are missing. Further phylogenetic analysis places the ones we found in the nuclear/chromatin remodeling family of Arps (Elmendorf et al., 2010). The most likely explanation for this result is that the actin/microfilament associated proteins in *Giardia* are functionally homologous but divergent in sequence compared to typical eukaryotic organisms.

Earlier research using heterologous antibodies found that actin mainly localized at the periphery of ventral disk region (Feely et al., 1982; Narcisi et al., 1994). In our laboratory, we developed polyclonal antisera raised in rabbit against bacterial recombinant *Giardia* actin protein. Using the rabbit sera, a single band of about 42 kd is recognized by Western Blot analysis. The size of this band is in agreement with the predicted result from the *Giardia* actin gene. Further, immunofluorescence assays reveal
that actin is distributed not only at the periphery of the ventral disk, but also under the plasma membrane and along the intracellular axonemes of the anterior flagella. Thus the localization of actin extends under the dorsal plasma membrane, in addition to the periphery of cell and lateral crest (Khoury and Elmendorf, unpublished). The distribution of actin to this new location not only explains the structural support of dorsal side of the parasites, but also suggests the possibility that the contraction of microfilaments in this region could change the shape of parasite to allow the necessary structural change for attachment.

To summarize, the microfilament system and microtubule system of Giardia are divergent. The Giardia microtubule system contains many known microtubule component proteins found in other eukaryotes, including all tubulin isoforms and several microtubule associated proteins, including the genes for microtubule-based motor protein dynein and kinesin. In contrast, the microfilament system is less clear: all known microfilament system proteins other than actin are missing. Both the microfilament system and the microtubule system are believed to have developed early in eukaryotic evolution; and they are usually highly conserved. It is unclear whether Giardia diverged from the eukaryotic lineage prior to the development of the full microfilament system or whether it lost many of the components.

1.5 Proposed mechanism of attachment

1.5.1 Previous research on attachment

Attachment is an important function for Giardia because without it Giardia cannot maintain an infection: peristalsis will clear free Giardia. Additionally, Giardia
have to be capable of detaching, swimming, and reattaching to sustain its presence within
the small intestine to avoid being excreted with continuous shedding of intestinal cells
(Elmendorf et al., 2003). Although much research has investigated the interaction
between Giardia and host epithelial cells and the mechanism of attachment, we still know
surprisingly little about the precise mechanism. Attachment is a dynamic process, such
that Giardia can switch from attachment to detachment rapidly. Some factors that can
influence attachment are temperature, pH, and ionic strength, and physiological
conditions are best for attachment (Gillin and Reiner, 1982). Attachment has been
observed on both biological (various mammalian cell lines) and inert (plastic and glass)
substrates (Knaippe, 1990), and the attachment force is insensitive to surface change or
hydrophobicity (Hansen et al., 2006). This indicates that the attachment process is not
dependent on a specific parasite-substrate interaction or even a charge attraction but is
likely to be the result of a biomechanical force.

Many studies of the interaction between Giardia and epithelial cells showed
direct contact via the ventral disk/ lateral crest and via the ventrolateral flange (Friend,
1966; Holberton, 1973a; Owen et al., 1979; Erlandsen and Chase, 1974; Erlandsen and
Feely, 1984; Chávez et al., 1986; McCabe et al., 1991; Sousa et al., 2001; Erlandsen et
al., 2004). In some TEM images, the lateral crest grasps the microvilli of epithelial cells
like hooks (Friend, 1966; Feely et al., 1984; Sousa et al., 2001). Especially in some SEM
images (Erlandsen and Chase, 1974; Owen et al., 1979; Chávez et al., 1986; Sousa et al.,
2001), “footprints” on infected epithelial cells correspond to the size and shape of lateral
crest left by previously attached Giardia. Thus these studies suggested the ventral disk
region plays an important role in attachment. But the exact mechanism of attachment remains unclear. Researchers have proposed some theories, and although all of them have some supporting evidence, the issue has not yet been completely dissolved (reviewed in Elmendorf et al., 2003).

1.5.2 The adhesion model of attachment

This model is also called the charge attraction model or the “sticking” model. Several groups found that the Giardia surface is negatively charged (Gonzalez-Robles et al., 1989). N-acetyl-D-glucosamine residues are present on the surface of Giardia, which can bind to receptors (lectin) on host epithelial cells (Farthing et al., 1986, Inge et al., 1988 and Ward et al., 1988). Evidence in support of this model comes from research showing that Giardia prefers to attach to intestinal epithelial cells of the small intestine compared to colon cells (Inge et al., 1988). Thus the specific interaction between parasites and host cells may result in the cell specificity. Generally it is believed that the lectin interaction may play a role in vivo to initiate an interaction between the parasite and host at the correct intestinal location but is not the most important force for attachment, as evidenced by the ability of Giardia to attach to a wide range of inert substrates with equivalent forces (Knaippe, 1990; Hansen et al., 2006).

Erlandsen et al. (2004) examined Giardia trophozoite attachment by allowing them to attach to a special microfabricated substrate with numerous pillars with smaller diameter than ventral disk. Thus the ventral disk and top surface of pillar cannot form a closed space for generating the hypothesized negative pressure. Giardia cells were mostly attached to the flat surface but a much lesser number (16% compared to control)
attached to the pillar region. The attachment on pillars seemed to be caused by adhesion between the ventrolateral flange and substrate. Erlandsen argued that the membrane adhesion of the ventrolateral flange may play a secondary role in attachment.

1.5.3 The grasping model of attachment

The ventral disk is such a unique structure that many researchers focus on the behavior of disk to understand the process of attachment. In 1974, Mueller proposed that the tightening of the spiral array of microtubules that make up the ventral disk surface should contract the ventral crest to clutch substrate surface (Mueller et al., 1974). Thus it was described as a clutching or grasping mechanism. However, microscopy studies demonstrated that the spirally-arranged microtubules in the disk are linked by filaments (Holberton 1973a), which cannot allow the movement between microtubules required in this model (reviewed in Elmendorf et al., 2003).

More recent proposals have suggested instead that a contraction at the periphery of the disk could deform the shape of the disk, changing its concavity. This model would fit with known ventral disk structural constraints and mirrors the change in disk shape observed between free swimming and attached parasites (reviewed in Elmendorf et al., 2003).

The actin detected at the periphery of the cell, lateral crest, and ventral disk, in addition to a network localized under the dorsal plasma membrane (Feeley et al., 1982; Khoury and Elmendorf, unpublished), suggests that the contraction of the fibrous matter in lateral crest region could provide force for the needed morphology change during
attachment (Friend, 1966; Sousa et al., 2001). However, directly investigating the function of cytoskeleton system is difficult due to our limited knowledge of *Giardia* cytoskeleton protein repertoire.

1.5.4 *The hydrodynamic model of attachment*

The continued beating of ventral flagella observed in attached *Giardia* has long suggested the possibility that negative pressure under the parasite – sufficient to promote attachment – may be generated by a fluid flow driven by the activity of flagella. Thus Holberton (1973b and 1974) and Soloviev (1968) proposed a hydrodynamic model of attachment. But the beating of ventral flagella is not only observed when parasites attach to substrate. The beating is continuous regardless of cell activity and therefore cannot account for the attachment and detachment dynamics (Elmendorf et al., 2003). Besides, due to an early incorrect observation of trophozoite morphology, Holberton’s biophysics calculations were based on an incorrect assumption that the ventral flagella emerged from the center of the disk. In fact later studies (Feely et al., 1984) revealed that the ventral flagella emerge from the cell body in the ventral groove, dorsal to the ventral disk.

1.5.5 *New observations*

In 2006, Hansen and colleagues indicated the *Giardia* attachment force is insensitive to different surfaces like positively charged hydrophobic substrate or inert surfaces (Hansen et al., 2006), which is more consistent with the suction based and clutching models. They suggest that lectin binding or adhesive mechanism is more likely to play secondary roles in attachment. In 2008, Hansen and Fletcher published a study
that revealed the detachment of *Giardia* is sensitive to the change of environmental osmolality (Hansen and Fletcher, 2008). Detachment can occur within 25 seconds after a tonic shock. The parasites that detach from the glass surface showed normal morphology and sinusoidal beating of ventral flagella. Thus the authors proposed a new mechanism that the osmotic pressure difference between a sealed ventral disk and environment generates attachment force.

### 1.6 Drug Studies of Attachment

#### 1.6.1 Summary of previous studies

Although the exact details of attachment are controversial, it is likely that the cytoskeleton plays an important role in attachment. Thus, many drug studies have focused on the disruption of these filaments to provide evidence for the participation of cytoskeleton system in attachment.

In 1982, Feely and Erlandsen first investigated the influence of the microtubule disruptor colchicine ($10 \, \mu g/ml$) and the microfilament disruptor cytochalasin-B ($10^{-4}, 10^{-6}$, and $10^{-8}$ M) on *Giardia* attachment. The result showed cytochalasin-B treatment dramatically decreases attachment rate by 40%, while colchicine had no significant effect on attachment (Feely and Erlandsen, 1982). McCabe et al.’s work (1991) verified the fact that microtubule-disrupting drugs have no effect. Further studies done with microfilament drugs, including cytochalasins-A,-B and –D, confirmed decreased *Giardia* attachment (Katelaris et al., 1995; Sousa et al., 2001). In contrasting studies (Magne et al., 1991; Katelaris et al., 1995; Sousa et al., 2001), researchers co-incubated *Giardia* with
epithelial cells and treated with either colchicine or nocodazole; they observed decreased attachment with both drugs compared to controls, although it is unclear whether the drugs were acting primarily on the parasite or host cells. Most studies did not check the cell viability or cytoskeleton ultrastructure when conducting drug treatments to examine the effect on their proposed targets. These may be the reasons why some studies yielded contradictory results.

1.6.2 Our recent research

To further investigate the role of cytoskeleton system, our laboratory performed in vitro attachment assays using different cytoskeleton drugs at a range of concentration to observe the effects on morphology and attachment to glass substrate (Khoury and Elmendorf, manuscript in preparation). The drugs were divided into two groups: drugs that affect the stability of either microtubules or microfilaments. Parasites were treated with drug solution or equivalent solvent concentrations (DMSO) as control. After different incubation times, the unattached cells were removed by decanting cultures. The remaining attached cells were chilled to detach and counted in a hemacytometer.

The microfilament-disrupting drugs, including cytochalasin-B, cytochalasin-D, and latrunculin-A, resulted in a statistically-significant decrease in attachment in a dose dependent manner. As expected, the actin staining pattern in treated cells showed disruption of peripheral microfilaments but no detectable change in microtubules was detected with anti-tubulin antibodies (data not shown). Cytochalasin-D and latrunculin-A were more effective than cytochalasin-B. Significant reduction of attachment with cytochalasin-D and latrunculin-A to 20% attachment was observed even at a low
concentration (2 \( \mu \text{M} \)) and as early as 30 minutes. By 240 minutes, only 5-10% attachment was observed when treated with cytochalasin-D and latrunculin-A, as well as less than 50% attachment for cytochalasin-B treatment.

In contrast, the microtubule disrupting/stabilizing drugs, including nocodazole, paclitaxel, and colchicine, showed a dramatic decrease in the length of microtubules and a subset of flagella as expected (data not shown), but attachment was largely unaffected. At low concentrations of 2 and 20 \( \mu \text{M} \), the attachment rates remained near 100%. Even at high concentration drug treatment, 200 \( \mu \text{M} \) for nocodazole and 5 mM for colchicine, the attachment rate is still above 40%. High concentration of paclitaxel (200 \( \mu \text{M} \)) did not affect attachment.

These results indicate a more important role for microfilaments than microtubules in attachment. Thus we will focus on the \textit{Giardia} actin and microfilament system for further research to elucidate the mechanism of attachment. To identify actin/microfilament associated proteins is the initial step.

1.7. Summary of Previous Microscopy Observations of \textit{Giardia}

Since Leeuwenhoek first observed \textit{Giardia}, microscopy techniques have been widely used in the investigation of the structures and behaviors of this tiny single celled parasite. A summary of the fine structures is listed in section 1.2.1. This section will concentrate more on the contribution of microscopic analysis to understand the biology and behaviors of \textit{Giardia}.

1.7.1 \textit{Plasma membrane observations by microscopy}
From light microscopy, TEM, and conventional SEM images, the surface of trophozoites is not complicated except at the ventral disk region, where flagella are emerging, and a curious depression on the dorsal surface. But when combining TEM with techniques such as fracture-fracture replicas and deep-etching, the surface of membrane revealed 10-15 nm diameter globular particles and some rugosities about 30 nm diameter on flagella membranes (Kattenbach et al., 1991; Kattenbach et al., 1996). Although the exact identifications of these particles are unknown, Souza and colleagues suggested the use of these methods to understand more about the nature of surface-associated macromolecules (Souza et al., 2004).

Another technique called freeze-fracture replicas was used to acquire information about the integral proteins on the plasma membrane. It was revealed the density of intra-membranous particles was higher on the cytoplasmic side than on the extracellular side (Kattenbach et al., 1991), which indicated specific organization of membrane in trophozoites. Cholesterol, a common component of eukaryotic membranes, was studied by adding filipin which can form detectable filipin-cholesterol complexes; cholesterol was observed by this technique to be less abundant on the membrane close to lateral crest region (Chávez et al., 1995), suggesting that the low cholesterol content in lateral crest membrane may provide more flexibility, which was consistent with the possible contraction motion along the periphery of the ventral disk.

1.7.2 Microscopy observation contributions to secretory pathway studies

A very powerful application of microscopy in *Giardia* research is to detect or identify organelles/proteins in the cell. One example is the peripheral vesicles that are
observed immediately under the dorsal membrane (reviewed by Friend, 1966; Adam, 1991). Although the composition and function of these vesicles were unknown for a long time, fluorescence microscopy, using small soluble molecules, such as lucifer yellow (internalized by endocytic process) and acridine orange (turns red in acidic environment) suggested these were similar to endosome. Lucifer yellow was found to accumulate in the peripheral vesicles, and the peripheral vesicles turned red after incubation with acridine orange (Lanfredi-Rangel et al., 1998). Because the lysosome marker acid phosphatase was also localized to the peripheral vesicles (Feely and Dyer, 1987; Kattenbach et al., 1991; and Lanfredi-Rangel et al., 1998), Lanfredi-Rangel et al. thus proposed the peripheral vesicles in *Giardia* represent an early endosomal-lysosomal system that further evolved into separate endosomes and lysosomes in higher organisms.

Another example is the search for the ER-Golgi complex in *Giardia* trophozoites. At first *Giardia* was believed to have no ER-Golgi complex. The development of microscopy techniques also made the identification possible. Not only were cisternae identified (Lanfredi-Rangel et al., 1998), two classic markers of eukaryotic ER cisternae, BiP and glucose-6-phosphatase were also co-localized with the cisternae like structures (Soltys et al., 1996; Lanfredi-Rangel et al., 1998). Although the presence of Golgi in *Giardia* is not fully resolved, microscopy techniques with more specific markers will greatly help these kinds of studies. Similarly, the investigation of encystation studies has been aided by microscopic analyses of the structures and processes involved in secretion through an elegant series of studies from the Hehl laboratory (e.g. Stefanic et al., 2006; Stefanic et al., 2009).
1.7.3 *The reproduction pattern observed by microscopy*

Mitosis is an important step in *Giardia* life cycle. In early research by light microscopy, a mother cell with four replicated nuclei was proposed to divide via dorsal-ventral equational way with each daughter cell receive one copy of each nucleus (Filice, 1952). The relative location of two nuclei would be unchanged during generations in this pattern. More recent research revealed that *Giardia* trophozoites divide with mirror-image symmetry in the plane of the adherence disc, thus called ventral-ventral equational division and the relative location of two nuclei will change with each generation, by using three-dimensional confocal microscopy and FISH technique to label one of the two nuclei (Ghosh et al., 2001). Later Yu et al. conducted similar research with FISH and confocal fluorescence microscope (Yu et al., 2002), but their data did not support the ventral-ventral equational division pattern because the location of the nucleus transfected with plasmid was maintained in *Giardia* clone over reproductions.

In 2006, Sagolla et al. finally shed light on this long-time puzzle by using three-dimensional deconvolution microscopy to observe each stage of mitosis, in which the mitotic spindles, the centromeres and the spindle poles were labeled by conserved cytological markers (Sagolla et al., 2006). The data revealed *Giardia* cytokinesis occurs along the longitudinal plane to ensure each daughter inherits one copy of each parental nucleus with mirror image symmetry.

1.7.4 *Microscopy observations for Giardia attachment*
The microscopic observations of *Giardia* trophozoites revealed close contact between the protruding disk on the ventral side to the substrate surface. In TEM images, the lateral crest or the outer rim of disk is usually found to ‘dig’ into the host microvilli like hooks, and the ventrolateral flange directly contacts microvilli (Erlandsen and Feely, 1984). The interference-reflection microscopy study that was done by the same group also suggested close contact by revealing dark regions in images corresponding to the lateral crest and ventrolateral flange (Erlandsen and Feely, 1984). Other microscopy studies concentrated on the microvilli damage caused by parasite attachment and revealed shortening, thickening, distortion and even removal of microvilli in the attachment area (Chavez et al., 1986; Koudela, 1994). But further investigation to distinguish between the respective roles of lateral crest and ventrolateral flange in attachment has not been done, partially due to their extreme close localization. Thus a sensitive enough method should be developed in attachment research to observe small spatial differences between structures in live cells.

Detachment has also been studied by microscopy methods, but the conclusions reached have been less complete. Erlandsen and Feely showed using time lapse and electron microscopy imaging that detachment is usually preceded by sharp upward flexion of the caudal flagella and posterior end (Erlandsen and Feely, 1984). A more recent study by Campanati et al. (2002) observed contraction of the ventral disk in free parasites undergoing attachment. But more detailed evidence of the detachment mechanism is still missing, and the connection between posterior end flexion and disk contraction needs further investigation.
1.7.5 *Microscopy observations for Giardia flagella*

The movement of trophozoites is described as ‘fallen leaf’ motility (Dobell, 1920), in which the cell body rotates around the longitudinal axis. Generally the flagella are believed to participate in the motility of trophozoites (Holberton, 1973b and 1974; Owen et al., 1980; Erlandsen and Feely, 1984; Gosh et al., 2001; Campanati et al., 2002). Recent technique such as the high frequency video-microscopy in which the time span between images can be less than the time required for one beating cycle for flagella made the detailed analysis of individual flagella movement possible (Gosh et al., 2001; Campanati et al., 2002; Elmendorf et al., 2003). During attachment, beating of ventral flagella via a sinusoidal pattern with the beating plane parallel to the ventral surface (Holberton, 1973a, b; Ghosh et al., 2001; Campanati et al., 2002). But the motions of the other flagella when cells are attached are not well understood. Some studies of attached parasites (Holberton, 1973a, b; Ghosh et al., 2001) mentioned the quiescent status of flagella except the ventral flagella, although other studies (Erlandsen and Feely 1984; Campanati et al., 2002) described fast beating of all flagella. This inconsistency in beating measurements in different studies may result from the differences in experimental conditions or differences in cell status, such as truly attached cells versus sliding cells.

During swimming, the ventral flagella are still beating similarly to attachment status. But the anterior flagella are beating in a different motion asynchronously with the angular beating plane relative to the longitudinal axis (Holberton, 1973a, b; Erlandsen and Feely 1984; Ghosh et al., 2001; Campanati et al., 2002). The motion of anterior flagella is described as ‘whip-like’ by Elmendorf et al. (2003) and researchers proposed
the specific swimming directionality is due to the asynchronous beating of anterior flagella (reviewed by Elmendorf et al., 2003).

1.8 Proposed Tools and Methodology

The aim of this research is to investigate the attachment machinery and mechanism of Giardia trophozoites. The studies were conducted from two perspectives: (1) the first approach is the identification of microfilament-associated proteins in Giardia lamblia by biochemical means. Because no other member of Giardia microfilament system is known other than actin, any finding will greatly improve our understanding of Giardia cytoskeleton and provides additional insight and tools for the study of the attachment process, and possibly contribute to future drug development. (2) The second approach is to understand the exact topology changes on the ventral side of attaching parasites using total internal reflection fluorescence (TIRF) microscopy. This observation will not only provide direct evidence to record the process of attachment, but also help validate the previously proposed attachment model or suggest a new mechanism of attachment, which will in turn contribute to fight against giardiasis by providing targets for interrupting attachment.

1.8.1 Co-sedimentation method

The principle of the co-sedimentation assay is the use of F-actin as bait to attract microfilament-associated proteins from a cell lysate of Giardia. For these assays we have used commercially-available human β-actin after numerous attempts to generate significant quantities of recombinant Giardia actin failed. We did not think it feasible
that our efforts would yield sufficient quantities of *Giardia* actin in the foreseeable future.

We believe human β-actin to be a suitable substitute for *Giardia* actin given the high conservation at both the primary sequence level (Figure 1.1) and the predicted conservation at the tertiary structure level (Figure 1.2).

To investigate the tertiary structure of *Giardia* actin, our collaborator, Stefano Costanzi, modeled the *Giardia* actin sequence onto a consensus mammalian β-actin structure. Overall, the tertiary structures of *Giardia* actin and mammalian actin are strikingly conserved except for changes in charge along a few pockets and loops (Figure 1.2). More importantly, the prediction of conserved structure and charge in many domains of human and *Giardia* actin argues for two points: (1) the fact that no canonical actin/microfilament associated proteins can be found in *Giardia* genome is likely not due to a difference in actin itself; (2) we should be able to use heterologous actin for actin binding studies as a substitute.

1.8.2 *Total internal reflection fluorescence microscopy method*

An important means to distinguish among the different models of *Giardia* attachment is the relative position of the ventral disk and other ventral structures to the substrate surface. In the ‘sticking’ or ‘clutching’ models, the edge of the lateral crest or ventrolateral flange should be the primary part of ventral side that directly contacts the substrate. It is also supposed for the ‘clutching’ model that the microtubule portion of the disk should become more upward arching to allow the lateral crest to grasp or clutch the substrate inwards (i.e. the microvilli of epithelia cells). Thus the height between ventral disk and substrate surface will change during attachment initiation. In the negative
pressure or ‘suction’ model, on the other hand, the cell should expel liquid below ventral disk to generate negative pressure, which involves displacing of the aqueous phase beneath ventral side. Thus some part of cell body may become closer to substrate surface to result in this expulsion and to establish attachment or flow of fluid under the ventral surface should be observable.

Although the plasma membrane of parasites can be labeled and observed by fluorescence microscopy, the accurate recording of the attachment process is not an easy task. First, because it is a dynamic process, the traditional high resolution technique of transmission electron microscopy can not be used because it applies only to static observations of fixed cells. Second, the distance between the ventral disk and substrate surface is quite small, measured only in hundreds of nanometers, thus the estimated distance change of cell membrane to substrate should be very subtle, and therefore impossible to capture by routine fluorescence microscopy. The total internal reflection fluorescence (TIRF) microscopy is an exact tool to overcome these handicaps.

When light passes through a plane separating two solutions with different refractive indices, refraction or reflection will happen according to the incident angle and the relative refractive indeces. The relation between refractive indeces and angles can be determined by Snell’s law:

\[ n(1) \cdot \sin(1) = n(2) \cdot \sin(2) \]

Thus, a specific angle called the critical angle will be reached when:

\[ \sin(c) = \frac{n(2)}{n(1)} \]
When the sine of the angle of refraction is less than 1, light is transmitted. Most importantly, when the sine of the angle of refraction is exactly 1, all light cannot transmit and travels at the plane between the two solutions (this incident angle defined as the ‘critical angle’). If incident angle is larger than critical angle, all light is reflected. However, a small portion of the light’s energy will create an electromagnetic field, called the evanescent field, along the interface plane that is capable of exciting fluorophores residing in the immediate region near the interface. The evanescent field intensity decreases exponentially with increasing distance from the interface; in practice, this is limited to excitation of fluorescence only up to several hundred nanometers from the interface. Thus TIRF microscopy can be used to detect even single labeled molecules within a 200 nm distance of the interface.

In TIRF microscopy, the fluorescence strength is a function of both the inherent brightness of the label and the distance between the position and reflection surface (i.e. the glass surface Giardia cells attach to): given even staining of a cell surface, the stronger the fluorescence, the closer the cell surface is to the substrate surface.
### 1.9. Figures

<table>
<thead>
<tr>
<th>Human</th>
<th>MDD0IAALVVDNSGMCKAGFAGGDAPRAVFPSIVGVRPHQGVMGQKDSYVGDEAQS 60</th>
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</thead>
<tbody>
<tr>
<td>Giardia</td>
<td>MTDONPAILVVDNSGMCKAGFAGGDAPRAVFPTVGRPKRETVLVGSTHHKEYIGDEAIA 60</td>
</tr>
</tbody>
</table>

**Figure 1.1: Sequence Alignment of Human β-actin and *Giardia* actin. ‘*’ indicates identical amino acids while ‘:’ indicate similar amino acids.**
Figure 2.1: The spatial structure and surface charge distribution comparison of human β-actin (top) and *Giardia* actin (bottom). The *Giardia* actin spatial structure is predicted based on modeling the *Giardia* actin sequence onto known crystal structure of mammalian β-actins. Both front and back view of human and *Giardia* actins are shown. Red indicates negative surface charge and blue indicates positive surface charge. Minor areas that have different charge distribution are indicated by yellow circles. Thus, the structure, topology, and surface charge distribution are similar for human and *Giardia* actins. This work was done in collaboration with Dr. S. Costanzi’s laboratory at National Institutes of Health.
CHAPTER 2

Identification of Microfilament-Associated Proteins in *Giardia lamblia*
Abstract

*Giardia lamblia* is a prevalent waterborne pathogen of humans, livestock, and pets. The attachment of the pathogen to the apical surface of small intestine epithelial cells establishes infection. While previous research has suggested an important role for microfilaments in attachment, recent bioinformatics analyses also reveal that *Giardia* has a single gene for actin but is missing the other ubiquitous eukaryotic microfilament proteins. To fully understand the function of microfilaments in the parasite, it is necessary to first determine their protein composition and localization. To identify possible microfilament-associated proteins in *Giardia*, we developed an actin co-sedimentation assay using purified human β-actin and *Giardia* cytosolic lysate followed by mass spectrometry. Our results show specific and significant enrichment of only *Giardia* actin and *Giardia*-specific proteins α-1 giardin and α-7.3 giardin, distant relatives of the larger annexin protein family. α-1 and α-7.3 giardins have been previously localized to the plasma membrane in *Giardia* and their homologs in other cells identified as binding to phospholipids and actin. We also localized actin using polyclonal antibodies raised specifically against recombinant *Giardia* actin and observe it under the dorsal plasma membrane, the periphery of the ventral disk, and the axoneme-associated structures of the ventral flagella. The localization of actin at the plasma membrane is new and suggests a possible functional interaction between actin and the α giardins in the parasite. Together, our data provide the first evidence for microfilament-associated proteins in *Giardia* and provide us with new leads to investigate microfilament function in the parasite.
Introduction

*Giardia lamblia* is a unicellular eukaryotic pathogen that causes a common diarrheal disease called giardiasis throughout the world (reviewed by Adam, 2001). Especially in developing countries where there is an insufficient clean water supply, it is a major contributor to diarrheal disease. The World Health Organization (WHO) estimates that more than 3 billion people are at risk, while 1 billion of them are infected annually (WHO/UNICEF, 2000). Even in the U.S., *Giardia* is a primary cause of waterborne outbreaks of diarrhea (Barwick et al., 2000). The annual reported incidence of giardiasis in the U.S. is about 20,000 (18,953 for 2006, Summary of Modifiable Diseases-United States, 2006; 19,417 for 2007, Summary of Modifiable Diseases-United States, 2007), with estimates of the actual incidence of infection between 2.0 and 2.4 million (Furness et al., 2000; Hlavsa et al., 2005) since most infected individuals are asymptomatic.

*Giardia* has two distinct life stages: environmentally-resistant, metabolically-quiescent cysts and vegetative trophozoites (Gillin et al., 1996; Lujan et al., 1997). Infection can begin by ingestion of as few as 10 cysts via contaminated water or food (Rendtorff, 1954). In the digestive tract of the host, excystation results in the transformation from cyst to trophozoite. During the trophozoite stage, the parasites attach to the epithelial cells of the small intestine to prevent premature passage and shedding, detach during parasite division, and reattach at a new site during epithelial cell shedding. The ability to attach to the wall of the digestive tract of host is therefore a virulence determinant in *Giardia.*
Although the attachment process has been a subject of study in the field for the past 30 years, we still do not understand much about the process or machinery involved. Data thus far indicate a biomechanical process unique among pathogen-host attachment process. Attachment is a dynamic process allowing *Giardia* to switch rapidly from attachment to detachment and back again.

Surprisingly, attachment has been observed to both biological (various mammalian cell lines) and inert (plastic and glass) substrates (Knaippe, 1990), with recent quantitative measurements indicating that the hydrophilicity / hydrophobicity / charge of the substrate does not significantly affect the force of parasite attachment (Hansen et al., 2006). Much work has focused on a role for the parasite’s cytoskeleton in attachment, with various studies over the years looking at microtubules or microfilaments. The more recent studies, including data presented here, clearly indicate a central role for microfilaments.

However our knowledge of the protein constituents of *Giardia* microfilaments is surprisingly limited (reviewed by Elmendorf et al., 2003 and 2010). The more noticeable structures of the parasite’s cytoskeleton, the eight flagella, ventral disk and median body, are primarily comprised of microtubules and affiliated structures, and evidence from the genome project suggests a full complement of tubulins, kinesins, dyneins, and microtubule-associated proteins (Morrison et al., 2007). In contrast, the localization of actin has been debated over the years with variable evidence for its presence distributed across a range of structures and cytoplasm, and, except for actin itself, no microfilament-associated protein has been identified in *Giardia* by sequence analysis (Morrison et al.,
Giardia actin has higher homology to eukaryotic non-muscle (β) actins than to muscle (α) actin and the comparison between Giardia actin and human β-actin reveals 60.5% identity and 79.2% similarity on the amino acid level, which is evenly distributed across the entire sequence. Strikingly, while Giardia actin was identified relatively early (Drouin et al., 1995), the expected actin binding proteins (ABPs), including the Arp2/3 complex, β-thymosin, cofillin, formin, gelsolin and profilin, and the myosins are not present. Importantly, earlier reports from indirect immunofluorescence assays using heterologous antibodies suggesting that the microfilament-associated proteins, α-actinin, tropomyosin, vinculin, and the motor protein myosin are present (Feely et al., 1982; Narcisi et al., 1994) are incorrect as evidenced by the absence of these genes in the Giardia genome database (Morrison et al., 2007; Elmendorf et al., 2010). Thus, despite their importance in general cellular dynamics; in addition to parasite attachment and virulence, we know almost nothing about the microfilament system in Giardia.

In our laboratory, we developed polyclonal antisera raised in rabbit against recombinant Giardia actin protein expressed in bacteria. Using the rabbit sera, a single band of about 42kd is recognized by Western Blot analysis of Giardia lysate, in agreement with the predicted size from the Giardia actin gene. Further, immunofluorescence assays reveal that actin is distributed under the plasma membrane, along the intracellular axonemes of the anterior flagella, and in the lateral crest of the ventral disk (Khoury and Elmendorf, unpublished). The distribution of actin to these new location not only explains the morphological support of the dorsal surface of the parasite, but also suggests the possibility that the contraction of microfilaments in this region
could change the shape of parasite to allow the necessary morphological change for attachment. Our lab thus developed a biochemical co-sedimentation method to identify *Giardia* actin/microfilament associated proteins that could be involved in such a function.

The focus of this paper is the development of an actin cosedimentation assay paired with mass spectrometry to allow us to directly identify microfilament-associated proteins in *Giardia*. Using this assay we have identified a subset of the α giardins as microfilament-associated proteins in *Giardia*, a group of proteins that also localize under the plasma membrane of the parasite.

**Materials and Methods**

**Giardia cell culture**

*Giardia lamblia* trophozoites (isolate WB1267) were maintained anaerobically (pH 7.5) in borosilicate glass tubes or polystyrene flasks (Fisher Scientific, 07-200-72). Parasites were grown at 37 °C in modified TYI-S-33 media (Keister, 1983), where the phosphate buffer was substituted with 0.024 M sodium bicarbonate. For all experiments, *Giardia* cultures were grown to mid-log phase (~ 80% confluency).

**Preparation of Giardia cytosolic lysate**

*Giardia* cultures were chilled on ice for 15 minutes to detach cells. Cells were collected by centrifugation at 1,200 x g for 10 minutes and washed three times with ice-cold PBS (50 mM potassium phosphate, 150 mM NaCl; pH 7.2). Cells collected from a single polystyrene triple flask (about 5×10^10 cells) were incubated with 4 ml lysis buffer
[10 mM HEPES, 1.5 mM MgCl$_2$, 10 mM KCl, 1% Igepal CA-630 (Octylphenyl-polyethylene glycol), 10 µM cytochalasin-D] on ice for 20 minutes. Finally the mixtures were centrifuged at 13,000 × g for 10 min at 4°C to clear nuclei and collect supernatant. This protocol is adapted from a nuclear isolation protocol with the modification that cytochalasin D was added, and the low detergent/low salt incubation is expected to keep the nuclei and other intracellular organelles largely intact, thus the lysate should be depleted in these cellular constituents. The purpose of the presence of cytochalasin-D is to maximize the release of microfilament-associated proteins from the cytoskeleton of cells. To avoid the influence of cytochalasin-D in the lysate on next co-sedimentation experiment, the cytochalasin D was removed from the lysate by dialysis in buffer containing 10 µM NH$_4$HCO$_3$ and 1 mM EDTA (pH 8.0) at 4°C for 24 hours.

**Polymerization and stabilization of F-actin**

One mg human non-muscle actin from platelets (Cytoskeleton, Inc.) was dissolved in 1 mL actin polymerization buffer [5 mM Tris (pH 8.0), 50 mM KCl, 2 mM MgCl$_2$, 1mM ATP] and incubated for 60 minutes. Dimethyl suberimidate was included in the incubation to 10 mM final concentration to chemically cross-link the polymerized actin. The polymerization of actin was verified by adding 5% pyrene labeled non-muscle actin (Cytoskeleton, Inc.) with non-muscle actin (Cytoskeleton, Inc.) in polymerization buffer (same as above) for detection of enhanced fluorescence of pyrene conjugates during the polymerization process. After polymerization, glycine was added to the incubation to a final concentration of 100 mM to quench the remaining dimethyl suberimidate. The reaction was centrifuged at 100,000 × g for 30 minutes at 4 °C to
collect the F-actin pellet. The F-actin pellet collected from 1 mg beginning G-actin was then resuspended in 200 µl low ionic strength buffer containing 2 mM Tris (pH 8.0), 0.2 mM CaCl$_2$, 0.2 mM ATP, and 0.5 mM DTT. The dissolved F-actin was kept on ice for next co-sedimentation steps.

**Co-sedimentation assay**

After thawing, *Giardia* cytosolic lysate was first centrifuged at 400,000 × g for 30 minutes to remove insoluble debris. The remaining lysate was collected and diluted in low ionic strength buffer [2 mM Tris (pH 8.0), 0.2 mM CaCl$_2$, 0.2 mM ATP, and 0.5 mM DTT] at a 1:3 ratio. Then 800 µL diluted lysate was mixed with 200 µL resuspended F-actin. After 1 hour incubation at room temperature on a rotator, the mixture was centrifuged at 100,000 × g for 30 min to collect the F-actin pellet. The supernatant from this step was further centrifuged at 400,000 × g for 30 min to collect other insoluble complexes. In control experiments, the lysate was mixed with low ionic strength buffer [2 mM Tris (pH 8.0), 0.2 mM CaCl$_2$, 0.2 mM ATP, and 0.5 mM DTT] alone.

Proteins from lysate, actin supernatant and pellet fractions were initially analyzed by one-dimensional SDS-PAGE on NuPAGE Novex 12% Bis-Tris gel (Invitrogen Inc.) according to protocol provided with SDS-PAGE Kit (Invitrogen Inc.). Samples were prepared for mass spectrometry assay by dialysis in buffer containing 10 mM NH$_4$HCO$_3$ and 1 mM EDTA (pH 8.0) at 4°C for 24 hours and then protein samples were lyophilized in a speed-vacuum.

**Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis**
This analysis was done in collaboration with Dr. Igor Almeida’s laboratory at University of Texas at El Paso. The samples were dissolved in 20 µL 0.4 M NH₄HCO₃ containing 8 M urea and the disulfide bonds were reduced with 5 mM DTT for 15 min at 50°C. After reduction, the free thiol groups were alkylated with 10 mM iodoacetamide for 30 min at room temperature, diluted 8-fold with HPLC grade water and digested for 16 hr with 2 µg sequencing grade trypsin at 37°C (Stone and Williams, 1996). The reaction was stopped with 1 µL formic acid (FA). Samples were desalted with POROS R2 ziptips (Applied Biosystems) and dried with a speed vac (Jurado et al, 2007). To remove the detergent present in the preparation, the peptides were purified in a strong cation-exchange (SCX) ziptip. Peptides were dissolved in 100 µL SCX buffer [25% acetonitrile (ACN)/0.5% FA] and loaded into SCX ziptip columns manufactured in Almeida’s laboratory using POROS HS 50 resin (Applied Biosystems). After loading the samples, the column was washed with 30 times the column volume of SCX buffer and the peptides were eluted with 50% ACN/200 mM NH₄OH (Cordero et al, 2009). Samples were then dried in the speed vac, resuspended in 30 µL 0.1% FA and subjected to 2D LC-MS system (LC – Eksigent 1D-plus, MS – Thermo Fisher LTQ XL with ETD, ESI source - Triversa, Adivion). To perform the LC-MS, peptides were loaded into in a lab-made C18 column (1 cm, 75 µm, Phenomenex Luna C18, 5 µm), whereas the separation was performed in a capillary C18 column (20 cm, 75 µm, Phenomenex Luna C18, 5 µm) in a linear gradient (Solvent A: 5% ACN / 0.1% FA, Solvent B: 80% ACN / 0.1% FA, 5-40% B in 600 min, 50-90% B in 1 min, 5 min in B, 90-5% B in 1 min and 20 min in 5% B). The MS system was set to perform one full scan (range: 400-2000 m/z) followed by
MS/MS of the 10 most abundant parent ions (isolation width: 3.0 m/z; normalized collision energy: 35%). The dynamic exclusion was set to collect each parent ion twice and then exclude them for 30 sec.

**Protein identification**

This analysis also was done in collaboration with Dr. Igor Almeida’s laboratory. The resulting MS/MS spectra (800-3500 Da, minimum of 10 counts) were converted to DTA files submitted to a database search using Sequest (available in Bioworks 3.3.1, Thermo Fisher Scientific) (Eng et al., 1994). The database was composed of *Giardia lamblia*, human keratin and actin, and porcine trypsin sequences (all in correct and reverse orientations) downloaded from GenBank ([http://www.ncbi.nlm.nih.gov/genbank/](http://www.ncbi.nlm.nih.gov/genbank/)) on August 28th, 2008. The parameters for the database search were: (i) 2.0 and 1.0 Da for peptide and fragment mass tolerance, (ii) trypsin cleavage at termini, and (iii) methionine oxidation, cysteine carbamidomethylation as possible modifications. The Sequest results were filtered in Bioworks with DCn $\geq 0.085$, protein probability $\leq 0.001$, and Xcorr $\geq 1.5, 2.2, 2.7$ for singly-, doubly- and triply-charged peptides, respectively. The false-discovery rate (FDR) was calculated to be approximately 1%.

**Statistical analysis of lysate**

This analysis was done in collaboration with Dr. Mahlet Tadesse at Georgetown University, Department of Mathematics. We used the descriptions/names to identify corresponding proteins between our data set and the genome database mass spectrometry
data set (http://giardiadb.org; Daniel M. Ratner, Michael Lubrano, Martin Steffen, John Samuelson. University of Washington, Department of Bioengineering). To simplify the analysis, proteins with the same name were collapsed into a single protein regardless of genomic location and the number of hits across all of the corresponding peptides were added up; this resulted in aberrant compression of only a very few, very large protein families (e.g. the variant-specific surface proteins) which were not particularly relevant to our analysis. For both data sets, the number of hits for each protein was normalized by the total number of hits in the corresponding assay. That is, the relative frequency of hits is used for each protein. For the genome data, we treated the data as though it was collected in a single experiment and used the same total count normalization approach.

We used one-sample \( t \)-tests (parametric test) and Wilcoxon tests (non-parametric test) to assess whether the location parameter of each protein in our lysate data differed from the standardized hit in the genome data. Given the small sample size, it is not possible to check the normality assumption required for the one-sample \( t \)-test. However, the Wilcoxon test is conservative. Since the test statistics are performed for each protein, we adjusted for multiple testing and used the Benjamini-Hochberg (BH) FDR approach (Benjamini and Hochberg, 1995).

**Statistical analysis of co-sedimentation samples**

This analysis also was done in collaboration with Dr. Mahlet Tadesse. The experimental samples consist of 5 replicates of total *Giardia* lysates, 5 replicates of control pellets with no exogenous actin added, and 5 replicates of co-sedimentation pellets with exogeneous (human) \( \beta \)-actin. To improve the reliability of our findings, the
replicates were performed with three different lysate preparations, a statistical complication we accounted for by analyzing the 5 replicates in the lysate group in three different batches representing the three different lysate preparations.

The data considered for analysis consisted of peptide hits recorded by mass spectrometry for each sample. Of the 1008 peptides recorded as having at least one hit in one of the experimental samples, 69 were not from *Giardia* and may have arisen from sample contamination, leaving 939 peptides for analysis. Due to the variations in experimental conditions and the variability in the amounts of material used, normalization of the data was required. A total count normalization approach was taken, in which every count $x_{ir}$ for a peptide $i$ in experiment $r$ is divided by $\sum_{j=1}^{r} x_{jr}$, the total number of counts in the corresponding mass spectrum. That is, the relative frequency of hits is used for each peptide.

Since the distribution of the data in each group is skewed, the non-parametric Kruskal-Wallis test was used to compare each peptide across the independent groups with same centrifuge speed. The test uses the ranks of the data to evaluate the equality of population medians across groups.

For the co-sedimentation and control groups, two pellets were collected: a 100,000 × g pellet that should contain microfilament-associated proteins and non-specific protein complexes, and a second 400,000 × g pellet that should contain only non-specific protein complexes. The differences of the attachment values within each pellet have a skewed distribution. Therefore, the non-parametric Wilcoxon signed-rank test was used.

The test statistics above were performed on each peptide, raising a problem of
multiple testing. Methods that control the family-wise error rate, that is, the probability of making at least one false rejection among all tests, lead to rejection rules that are too stringent when hundreds of hypotheses are evaluated. The FDR procedure developed by Benjamini and Hochberg controls the proportion of errors among rejected tests and provides a less conservative approach.

The open source software R (http://cran.r-project.org/) was used for all data processing and analysis.

**Results**

**Using human β-actin as a substitute for *Giardia* actin**

In order to identify microfilament-associated proteins in *Giardia*, we performed a co-sedimentation assay and screen *Giardia* lysate by using F-actin as bait to attract possible actin-binding proteins, collect these proteins by differential centrifugation, and identify by mass spectrometry analysis. Despite repeated efforts, we were unable to purify sufficient quantities of recombinant *Giardia* actin using a baculovirus expression system (data not shown). Likewise, isolation of a sufficient quantity of highly-purified actin from *Giardia* lysate is not technically feasible based on parasite yields from culture because of the relatively low abundance of actin in *Giardia*. We have succeeded in purifying substantial quantities of recombinant actin from an *E. coli* expression system, but expression in a prokaryotic system is likely to result in incorrect folding of actin, so while this recombinant protein has proven useful for production of anti-actin antibodies, we did not deem it appropriate for molecular interaction assays.
Because *Giardia* actin demonstrates strong homology at a sequence level to β-actins of mammals, the spatial structure of *Giardia* actin was predicted by modeling the *Giardia* actin sequence onto the consensus crystal structure of mammalian β-actins (Figure 2.1: This work was done in collaboration with Stefano Costanzi’s laboratory at National Institutes of Health). A comparison of the structural homology model of *Giardia* actin with mammalian β-actin demonstrates very high conservation of protein structure, particularly on faces of the proteins known to be involved in interaction with actin-binding proteins (S. Costanzi, personal communication; data not shown). The high level of structural similarity with *Giardia* actin suggests that it is likely to serve as an appropriate substitute in co-sedimentation assays and should be able to bind effectively to a range of *Giardia* actin-binding proteins.

**Preparation and stabilization of F-actin**

The extent of human β-actin polymerization was monitored by fluorescence generated from pyrene labeled actin during the polymerization process. We were able to readily generate human β F-actin microfilaments under standard polymerization conditions (Figure 2.2).

We next investigated the stability of these human β F-actin filaments during our co-sedimentation assay conditions, expecting that high-speed centrifugation, changes in buffer composition, and association with actin-binding proteins might alter the filaments. To test this, we carried out F-actin co-sedimentation experiments in actin polymerization buffer, monomeric actin maintenance buffer, or mixed with *Giardia* lysate. The results revealed that F-actin is most stable in actin polymerization buffer, and least stable in in
low ionic strength buffer (Figure 2.3). While it appears that some proteins in *Giardia* lysate may contribute to the stability of F-actin via a possible interaction with actin filaments, we sought to improve the stability of F-actin during incubation with *Giardia* lysate. To do this, we added dimethyl suberimidate, a standard chemical cross-linker for the stabilization of F-actin (Ohara et al., 1983), during F-actin preparation (data not shown). To inactivate the cross-linker and prevent aberrant cross-linking by dimethyl suberimidate of *Giardia* proteins to the F-actin, glycine was added to react with extra free dimethyl suberimidate and the F-actin was collected as a pellet by centrifugation.

**Preparation of *Giardia* lysate**

Since our primary interest was the identification of cytosolic *Giardia* proteins capable of binding to actin, we prepared parasite lysates that did not include nuclear fractions and eliminated large cytosolic complexes through a pre-centrifugation step that might contaminate a co-sedimentation assay. The protein composition of these lysates was compared to the only publicly-available mass spectrometry data set for *Giardia* that is available on the *Giardia* genome database ([http://giardiadb.org](http://giardiadb.org)) which was generated using the same strain of *Giardia* and similar, although not identical growth conditions (differing as far as we know only in their use of a phosphate buffer and our use of a bicarbonate buffer to maintain culture pH). The prominent difference between the two samples was our selection of only soluble cytosolic proteins while they aimed for a total protein fraction by extracting proteins from parasites using 8 M urea and 0.05% SDS.

Statistical comparison of the two sets of mass spectrometry data yielded 1,302 proteins in the genome mass spectrometry data set and 709 proteins in our lysate. Of
these, 566 were common to both data sets and were considered for further investigation. 143 proteins in our lysate preparation are not found in the genome mass spectrometry data set, and 160 proteins found in the genome mass spectrometry data set are not found in our lysate. Further analysis of the identity of the proteins missing in our lysate indicates that a large number are nuclear proteins or integral plasma membrane proteins that we would expect to be missing from our lysate because of the method of preparation (data not shown). The abundance of membrane and cytosolic proteins usually are highly correlated (e.g. high cysteine membrane protein Group 1, Bip, and arginine deiminase). We saw decreased abundance of cytoskeleton proteins that are tightly bound to highly stable cytoskeleton structures – likely to be removed from our lysate by the pre-centrifugation step to clear the lysate – such as axoneme-associated protein GASP-180, median body protein and arginyl-tRNA synthetase. However, many cytoskeleton proteins – mostly microtubule-associated proteins since these are the only ones identified in Giardia – are retained in our samples, suggesting that actin-associated proteins should be present, too. Overall, these data support the appropriateness of our lysate samples for use in our co-sedimentation assay.

**Purification of Giardia proteins in F-actin co-sedimentation assay**

In order to identify actin-binding proteins in Giardia, we established an F-actin co-sedimentation assay to isolate candidate Giardia proteins from Giardia cytosolic lysate. In this assay, we used the human β-actin F-actin filaments described above and Giardia lysate prepared in previous steps. We conducted actin co-sedimentation experiments with parallel control co-sedimentation experiments that differed only in the
omission of F-actin from the experiments. Protein samples were collected and analyzed by one-dimensional SDS-PAGE.

The results from SDS-PAGE demonstrated stable maintenance of F-actin and revealed several contaminating human proteins are present in the commercially-prepared β-actin (ActinP and Pellet lanes from Figures 2.4 and 2.5). The centrifugation step to pre-clear the lysate shows the importance of this step to remove the numerous Giardia proteins present in lysate pellet (Lysate and LysateP lanes from Figure 2.5).

The majority of the F-actin added to the Giardia lysate was collected in pellet 1 at 100,000 × g, along with numerous Giardia proteins (Pellet1 lane from Figure 2.4). In contrast, in pellet 2 samples collected at 400,000 × g, the amount of F-actin was dramatically reduced, while the abundance of Giardia proteins was not significantly diminished (Pellet 2 lane from Figure 2.4), suggesting non-specific aggregation and sedimentation should characterize the proteins found in pellet 2. Comparing pellet samples between the actin co-sedimentation and control co-sedimentation samples revealed that most Giardia proteins presenting in the pellets are also present in control pellets (CPellet lane from Figures 2.4), suggesting that non-specific aggregation of many Giardia proteins is a source of many Giardia proteins in both pellet 1 and pellet 2 fractions.

Mass spectrometry identification of microfilament-associated proteins

In total 5 actin co-sedimentation samples and 5 control sedimentation samples were analyzed by mass spectrometry. Both actin co-sedimentation and control sedimentation samples contained 100,000 × g pellets (pellet 1) and 400,000 × g pellets
(pellet 2). Human β-actin and other protein contaminants (e.g. keratin) were present in all samples and eliminated from original dataset prior to further analysis.

To identify *Giardia* microfilament-associated proteins, we focused on three comparisons: (1) comparison of protein abundance between actin sedimentation experiments and control (no actin) sedimentation experiments for both the 100,000 × g pellets and 400,000 × g pellets, (2) comparison of protein abundance between 100,000 × g pellets and 400,000 × g pellets within samples, and (3) overall enrichment of protein abundance in co-sedimentation samples v. total *Giardia* lysate. If the enrichment of a protein during co-sedimentation is actin-dependent, we would expect it to be more abundant in actin sedimentation pellets compared to control pellets. Likewise, our SDS-PAGE results indicated that the 400,000 × g pellets from the co-sedimentation assays contained little actin but included many *Giardia* self-aggregating protein complexes. An overall enrichment of specific proteins should be a hallmark of any purification protocol. This suggests that pellet 1 from the actin co-sedimentation assays should be our primary source of microfilament-associated proteins.

We identified a total of 709 proteins in our *Giardia* lysate samples. In comparing the actin-cosedimentation v. control cosedimentation 100,000 × g pellet protein profiles, we find that only 8 *Giardia* proteins are selectively enriched in the actin-cosedimentation pellet with a cut-off P<0.2 (Table 1). The statistical analysis is based on the median concentration of individual proteins; we chose to compare median value and permit a relatively high P value because of the variation observed between experiments performed with different lysate preparations (discussed later). We then used the difference in
relative abundance between the 100,000 × g pellets and 400,000 × g pellets to eliminate non-specific self-aggregating proteins. In Table 2, we show the top proteins with actin dependent enrichment in 100,000 × g samples as ranked by their P value. Using P<0.2 as the cut-off to determine significant enrichment, we find that only 3 proteins meet our criteria: *Giardia* actin, α-1 giardin and α-7.3 giardin. Additionally, while not meeting our criteria for significance, we note that the next proteins that appear to have a specific association with actin are α-6 giardin, α-2 giardin, and a VSP.

**Discussion**

Our inability to identify microfilament proteins other than actin in *Giardia* by bioinformatics analysis of genome data suggests that any microfilament-associated proteins in *Giardia* are highly divergent at the sequence level even if they serve similar functions as the traditional eukaryotic microfilament proteins. Thus, we took a biochemical approach to purification and identification of microfilament-associated proteins in *Giardia*. In the study described here, the interaction between *Giardia* proteins and polymerized human β-actin in a co-sedimentation assay was chosen as a criterion to identify possible microfilament-associated proteins in *Giardia*.

The goal of our preparation of *Giardia* lysate was to eliminate irrelevant proteins and structures that might co-precipitate with actin in our co-sedimentation assay while retaining as many cytosolic proteins as possible. Thus the extraction conditions we used were relatively gentle and designed to preserve intact nuclei. Mass spectrometry data indicate that our resultant lysate largely met our criteria and appeared predominantly as a
mixture of cytosolic, organellar, and plasma membrane proteins while nuclear proteins and flagellar and median body proteins are scarce. Overall the proteomic repertoire of our *Giardia* lysate was very complex and contained 709 *Giardia* proteins.

In actin co-sedimentation experiments, we typically identified fewer than 100 *Giardia* proteins in the 100,000 × g pellets. Many of these are undoubtedly contamination of either abundant parasite proteins or self-aggregating parasite proteins. In the co-sedimentation experiments it was necessary to modify the ionic conditions of the lysate to stabilize actin polymers. Yet many other proteins in the lysate may also aggregate under these conditions, and this appears to be a primary reason for the non-specific presence of *Giardia* proteins in the 100,000 × g pellets of the actin co-sedimentation experiments. We used two controls to enable us to identify specific microfilament-associated proteins. The first is the use of control co-sedimentation experiments in which F-actin was omitted. The second is the examination of a 400,000 × g pellet from each sample. Together these controls should account for anomalous proteins present only because of their sheer abundance or because of their actin independent aggregation properties. True microfilament-associated protein candidates should show significant difference between actin sedimentation and control experiments with a reasonable P value. Usually P<0.05 is chosen as the threshold to avoid false positives (type I errors). The proteins listed in table 1 and 2 have raw P values of P<0.05. However, to account for the multiple comparisons used in the statistical analysis of these data and to control the false discovery rate, we have chosen to use the more appropriate BH adjusted P values. For the BH adjusted P values, all proteins failed to satisfy the requirement P<0.05. Even *Giardia actin*, listed as
the top hit on both tables, the adjusted P value is 0.082 and 0.181 for table 1 and table 2, respectively.

We have therefore chosen P<0.2 as a stringent alternative BH-adjusted P value to minimize false negatives (type II error). At this level of significance, Giardia actin, α-1 giardin and α-7.3 giardin (all with BH-adjusted P values of 0.181) are the only specific microfilament-associated proteins we identify as enriched in both actin-containing samples relative to controls and in 100,000 × g pellets relative to 400,000 × g pellets.

The position of actin at the top of the lists appears to validate our co-sedimentation experiments and argues that the relatively high BH adjusted P values we observe are due to the relatively high variance between different experiments. There are many factors that contributed to the variation. The batches of commercially-obtained human β-actin used in different co-sedimentation experiments varied as determined by the presence of additional proteins on one-dimensional SDS-PAGE (data not shown). Additionally, we prepared different batches of Giardia lysate for use in different co-sedimentation experiments. Although these variations proved challenging for our statistical analyses, we believe that the relative consistency observed across the experiments provided important independent support for the reliability of our data.

According to SAGE data and mass spectrometry analysis based on whole cell composition from Giardia database, the abundance of actin in Giardia is relatively low. In this study, mass spectrometry analysis revealed that sometimes Giardia actin was not detected in the cytosolic lysate samples. Thus the abundance of Giardia actin in human β-actin pellet sample represented a significant association. Previous studies reported the
copolymerization of muscle actin and parasite actin such as *Tetrahymena* and *Acanthamoeba* (Hirono et al., 1990; Gordon et al., 1976). Although not been reported previously in *Giardia*, it is reasonable to suppose that *Giardia* actin can co-polymerize with human actin due to high level of similarity. We plan to specifically explore the nature and extent of this co-polymerization in future research.

The function and localization of α-giardins provide indirect evidence to support the interaction observed in our study. Giardins are a very diverse group of cytoskeleton proteins (Crossley and Holberton, 1983a, b) and can be subcategorized into α, β, γ, and δ giardins. The α-giardins are a very large family of proteins in *Giardia* with 21 members that are related to annexins and are highly immunogenic (Weiland et al., 2005). In this research, only α-1 and α-7.3 giardins showed enrichment in actin co-sedimentation experiments; α-2 and α-6 giardins also showed a dramatic enrichment but the increase missed our significance cut-off (Tables 1 and 2).

Annexins are a family of proteins of which many of them can bind to phospholipids and cellular membranes in a Ca$^{2+}$-dependent manner; they are functional in many membrane-related processes including cytoskeletal rearrangements and membrane organization (Gerke and Moss, 2002). Although the sequence of α giardins and even crystal structures of calcium-bound α-11 giardin and α-14 giardin are known (Pathuri et al., 2007 and 2009), the exact biological function of α giardins is poorly understood. It is believed, based on co-localization studies, that at least a subset of the α giardins may participate in cytoskeleton system in *Giardia* (Weiland et al., 2005). In Weiland and colleagues’ studies, AU-1 tagged α giardins were expressed in *Giardia lamblia* and
localized the distribution of various α giardins. Among these giardins, α-1, α-2, α-7.2, α-7.3 localize to the plasma membrane. The similar distribution of actin under plasma membrane that we observe provides the possibility of cellular interaction between actin and α-1 and α-7.3 giardins in vivo.

Interestingly, α-2 and α-6 giardin associated with actin preferentially in our co-sedimentation assay, although they missed the significance cut-off. α-2 also localizes to the plasma membrane and α-6 shows spotty localization in the cytoplasm ((Weiland et al., 2005). According to the phylogenetic tree of α giardin family tree provided by Weiland’s work, there is high homology among α-1, α-2, and α-6 giardin. In contrast, α-11 giardin, an abundant giardin whose localization is undetermined (overexpression of AU-1 tagged α-11 giardin was lethal), is always present in the 400,000 × g pellet but not in 100,000 × g pellet. Thus its behavior seems to be opposite to α-1, α-2, α-6 and α-7.3 giardins and suggests a specificity for our observed interactions.

To summarize, the identification of specific members of α giardins as the actin associated proteins in Giardia is consistent with our initial hypotheses that the proteins comprising the Giardia microfilament system except actin itself should be functional substitutes, but not sequence homologues to known actin associated proteins in other eukaryotic organism. Further support comes from the fact the identified actin-related proteins in Giardia, previously postulated to be more closely related to the nuclear protein within the Arp family (Elmendorf et al., 2010) do not associate with actin in our assays, suggesting that they are not distant homologues of the Arp1, 2 or 3 proteins.
Future work will be required to test the interaction between α-1 and α-7.3 giardins with *Giardia* F-actin by co-sedimentation or immunoprecipitation. Also, more detailed information about crystal structures of these specific α giardins may better indicate the functional residues for interaction with actin. Recently, *Giardia* actin was chosen as a new target for development of anti-*Giardia* drugs because attachment and encystation can be disrupted using microfilament-distrupting drugs (Castillo-Romero et al., 2009 and Elmendorf et al., 2010), which can in turn reduce infection and transmission. Similarly, the giardins, which may play a role in microfilament dynamics, can provide new drug targets. Due to the uniqueness of giardins within *Giardia*, such drug development directions should minimize the possible impairment to host cells and have clinical application.
Table 2.1: The list of the top 12 *Giardia* proteins that were enriched in F-actin pellets collected from 100,000 × g centrifugation step of actin co-sedimentation experiments (5 replicates). Proteins are ranked by the P-value [Benjamini-Hochberg (BH) adjusted P values] from low to high. Mean column and median column represent the mean value and median value for relatively abundance of each protein based on the statistic analysis of mass spectrometry protein identification data from actin co-sedimentation experiments, respectively. The enrichment of protein was determined by the comparison of relative abundance between the actin co-sedimentation experiments and control experiments.
Table 2.2: The top 12 *Giardia* proteins that were F-actin-dependent enriched in F-actin pellets collected from 100,000 × g centrifugation step of actin co-sedimentation experiments (5 replicates). Proteins are ranked by the P-value [Benjamini-Hochberg (BH) adjusted P values] from low to high. Mean pairdiff column and median pairdiff column represent the difference of mean value or median value for relatively abundance of individual protein between the pellets collected from 100,000 × g centrifugation step and 400,000 × g centrifugation step of actin co-sedimentation experiments or control experiments, respectively. If the abundance difference was higher in actin co-sedimentation data, the enrichment was more likely to be F-actin depended.
Figure 2.1: The spatial structure and surface charge distribution comparison of human β-actin (top) and *Giardia* actin (bottom). The *Giardia* actin spatial structure is predicted based on modeling the *Giardia* actin sequence onto known crystal structure of mammalian β actins. Both front and back view of human and *Giardia* actins are shown. Red indicates negative surface charge and blue indicates positive surface charge. Minor areas that have different charge distribution are indicated by yellow circles. Thus, the structure, topology, and surface charge distribution are similar for human and *Giardia* actins. This work was done in collaboration with Dr. S. Costanzi’s laboratory at National Institutes of Health.
Figure 2.2: The fluorescence enhancement of pyrene conjugates during actin polymerization process. The fluorescence signal increases during the initial incubation stage and reaches a plateau after about 4 minutes.
Figure 2.3: The comparison of stability of F-actin in actin polymerization buffer [5 mM Tris (pH 8.0), 50 mM KCl, 2 mM MgCl$_2$, 1mM ATP], low ionic strength buffer [2 mM Tris (pH 8.0), 0.2 mM CaCl$_2$, 0.2 mM ATP, and 0.5 mM DTT] and *Giardia* lysate [lysing buffer: 10 mM HEPES, 1.5 mM MgCl$_2$, 10 mM KCl, 1% Igepal CA-630 (octylphenyl-polyethylene glycol)] by SDS-PAGE. F-actin was prepared from human β-actin and the F-actin was collected by 100,000 × g centrifugation (30 min). Then F-actin was added into each solution and the actin still in the F-actin form was collected by 100,000 × g centrifugation (30 min) after 1 hour incubation on shaker. Both pellet and supernatant were collected and 10% of the pellet or supernatant samples were loaded onto an SDS-PAGE gel for comparison of actin abundance. The protein bands were shown by silver staining according to the protocol provided by the Pierce silver staining kit (Thermo Scientific, Inc.). L: ladder; AS: actin supernatant from centrifugation step that collect F-actin from starting materials; AP: F-actin collected from starting materials by centrifugation; 1S: supernatant of incubation with F-actin and actin polymerization buffer; 1P: pellet of incubation with F-actin and actin polymerization buffer; 2S: supernatant of incubation with F-actin and low ionic strength buffer; 2P: pellet of
incubation with F-actin and low ionic strength buffer; 3S: supernatant of incubation with F-actin and *Giardia* lysate; 3P: pellet of incubation with F-actin and *Giardia* lysate.
Figure 2.4: The comparison between samples from co-sedimentation experiment and control experiment by SDS-PAGE. The sample that was loaded onto each lane of an SDS-PAGE gel represented 10% of the total sample. The protein bands were shown by silver staining according to the protocol provided by the Pierce silver staining kit. Actin: starting actin material; ActinS: supernatant sample collected after 100,000 × g centrifugation step of collecting F-actin from starting actin material; ActinP: F-actin collected from starting actin materials by 100,000 × g centrifugation; Lysate: original cytosolic lysate; LysateP: lysate debris that was collected from original lysate by 100,000 × g centrifugation; Pellet: pellet collected from 100,000 × g centrifugation of actin co-sedimentation experiment; CPellet: pellet collected from 100,000 × g centrifugation of control experiment; FinalS: final supernatant sample collected after 100,000 × g
centrifugation step of actin co-sedimentation experiment; CFinalS: final supernatant sample collected after $100,000 \times g$ centrifugation step of control experiment.
Figure 2.5: The analysis of protein samples from co-sedimentation experiments by SDS-PAGE. The sample that was loaded onto each lane of an SDS-PAGE gel represented 10% of the total sample. The protein bands were shown by silver staining according to protocol provided by Pierce silver staining kit. Actin: starting actin material; ActinP: F-actin collected from starting actin materials by 100,000 × g centrifugation; Lysate: original cytosolic lysate; LysateP: lysate debris that was collected from original lysate by 100,000 × g centrifugation; Pellet1: pellet collected from 100,000 × g centrifugation of actin co-sedimentation experiment; Pellet2: pellet collected from 400,000 × g centrifugation of actin co-sedimentation experiment; FinalS: final supernatant sample collected after 100,000 × g centrifugation step of actin co-sedimentation experiment.
CHAPTER 3

Analysis of *Giardia* Attachment by TIRF Microscopy Support a Negative-Pressure Model of Attachment
Abstract

*Giardia lamblia* is a protozoan parasite responsible for widespread diarrheal disease in humans and animals worldwide. The parasite’s ability to attach to the host intestinal mucosa is necessary for establishing infection, but how *Giardia* effects this attachment is not well understood. Here we use total internal reflection fluorescence (TIRF) microscopy to study the topology of attachment between parasites labeled with Alexa-488 conjugated wheat germ agglutinin and a glass substrate. TIRF microscopy allows for a more precise visualization of the plane of attachment than has previously been possible, and our findings provide intriguing insights into the biomechanics of attachment. TIRF images reveal that the center of the ventral disk (a region termed the bare zone), the edge of the ventral disk (a region termed the lateral crest), and the posterior body of the parasite flanking the ventral groove (a region we term the lateral shields) are in closest apposition to the substrate, with the bare zone showing the first and most dramatic change in morphology during attachment and detachment. We additionally have used fluorescent microspheres to document the presence of fluid flow under the surface of the ventral disk. Together, these observations provide biophysical support for a negative pressure model of attachment.
**Introduction**

*Giardia lamblia* is a unicellular eukaryotic organism that causes a worldwide diarrheal disease called giardiasis. Importantly, *Giardia* parasites attach to the intestinal epithelial mucosa, and this behavior is therefore important to establish the infection (Wolfe, 2000; Sanchez et al., 2008). *Giardia* cells are relatively small – approximately 12-15 µm long and 5-9 µm wide – and are highly polarized with a well-developed and unique cytoskeleton (Feely et al., 1984; Elmendorf et al., 2003; Elmendorf et al., 2010). The parasites have eight flagella, two nuclei, and a cytoskeleton structure of unknown function termed the median body. The broader end of their flattened pear-shape is the anterior part of the cell, dominated on the ventral surface by the ventral disk, a structure unique to the genus. The ventral disk is comprised of a counter-clockwise (when viewed from the ventral side) spiral array of microtubules just under the plasma membrane. The microtubules originate from undefined structures between the two nuclei of the parasite and coil one-and-a-half times around to form a bowed disk shape around a center area cleared of microtubules – a region termed the “bare zone” – while at the periphery of disk, a network of fibers termed the “lateral crest” replace microtubules. On their ventral surface, the microtubules are connected to the plasma membrane by undefined fibers; while on their dorsal surface, highly ordered “dorsal ribbons” extend in an interconnected fashion. The cell body extends in a soft fold beyond the edge of the ventral disk in the anterior half of the parasite, creating a region known as the “ventrolateral flange”, while the space between the ventrolateral flange and lateral crest of the disk is termed the “marginal groove”. Because of the tight apposition between the anterior ventral side of
the parasite and substrate, it has long been postulated that the ventral disk is important for parasite attachment, although its precise function has never been elucidated.

The proposed mechanisms of attachment can be grouped into 4 categories (reviewed in Elmendorf et al., 2003; Elmendorf et al., 2010): (1) A ‘sticking’ model that proposes lectin-sugar interactions or charge attractions as the mediator of attachment (Farthing et al., 1986; Inge et al., 1988; Ward et al., 1988); (2) A ‘sucking’ model that postulates the generation of negative pressure beneath the ventral disk (Friend, 1966; Sousa et al., 2001); (3) A ‘clutching’ model that suggests a role for contractile proteins around the lateral crest or ventrolateral flange to enable the cell to grasp a substrate (Friend, 1966; Sousa et al., 2001); (4) A tonicity model that is proposed by Hansen et al. suggests some mechanism triggered by tonicity change in environment allow trophozoits attach or detach from substrate (Hansen et al., 2006).

Research in the field over the past 40 years agrees on the fact that attachment is a dynamic process, requiring viable parasites (McCabe et al., 1991; Sousa et al., 2001), ATP (Feely and Erlandsen, 1982), warm temperatures (Gillin and Reiner et al., 1982). Beyond this, however, there is little consensus (reviewed by Elmendorf et al., 2003). While several lines of evidence suggest that lectin/sugar interactions are likely to play a role in vivo, and research has revealed that the lateral crest and ventrolateral flange contact the host epithelium during attachment (Erlandsen and Feely, 1984; Koudela, 1994), Hansen and colleagues have recently shown that the *Giardia* attachment force is insensitive to charged, hydrophobic and inert surfaces (Hansen et al., 2006). Thus while the ‘sticking’ model may play a contributory role in attachment, it is neither necessary
nor sufficient. Likewise, a source for the negative pressure in the ‘suction’ model has eluded researchers, though at various times roles have been suggested for the beating of the ventral flagella or the distortion of the ventral disk as observed upon attachment. The ‘clutching’ model is supported by observations of the lateral crest seemingly digging into the surface of intestinal epithelial cells and the footprints of the ventral disk left behind after parasites detach (Erlandsen and Feely, 1984).

One key means to distinguish between these different models of *Giardia* attachment is the position of various parasite structures on the ventral surface of the parasite relative to the substrate surface. In the ‘sticking’ or ‘clutching’ models, we would expect only the edge of the ventrolateral flange or lateral crest, respectively to tightly contact the substrate. Correspondingly, in the ‘clutching’ model the concave shape of disk should become more pronounced to allow the lateral crest to dig into the substrate (i.e. the microvilli of epithelia cells or microscopic imperfections in an inert substrate), and the space between the center of the ventral disk and substrate surface would increase. In the ‘suction’ model, on the other hand, the cell would need to expel fluid from below the ventral side to generate negative pressure, and in turn the negative pressure would act to pull the parasite closer to the substrate.

Previous research to examine the regions of contact between the parasite and substrate has been surprisingly limited. Most work has been done on non-living cells: TEM and SEM images of parasites attached to a wide range of intestinal epithelial surfaces. Here there is good consensus that the lateral crest is in close contact with the microvilli, leaving behind a clear ‘footprint’ – an imprint of the ventral disk that often is
clear enough to see a negative image of the spiral shape of the disk in a region of microvilli shortening (Erlandsen and Chase, 1974; Chavez et al., 1986 and Sousa et al., 2001). These studies also demonstrate a contraction of the lateral crest to ‘dig into’ the epithelial cell surface (Erlandsen and Feely, 1984), and an increase in concavity of the ventral disk upon attachment (Sousa et al., 2001). Because these studies can capture only a static image of attached parasites, their use in understanding the mechanism of attachment is unfortunately limited.

A few studies have examined attachment in living parasites. Striking interference-reflection microscopy (IRM) images taken over 25 years ago by Feeley and Erlandsen in 1984 showed tight contact between the parasite and a glass substrate primarily along the lateral crest and the ventrolateral flange, although the level of resolution in the z-plane afforded by IRM is much less than that available by more modern microscopy techniques. Ghosh and colleagues (2001) examined the flow of beads under attached parasites but unfortunately did not show any data to accompany their observations. High-resolution video imaging of attached parasites suggests that contraction of either the anterior or posterior end of the disk occurs upon detachment (Campaneti et al., 2002).

In this research we chose total internal reflection fluorescence (TIRF) microscopy as a precise tool to provide direct evidence for the dynamics of the Giardia-substrate interaction by recording the topology and indirect measurements of the distance between membrane and substrate during the attachment and detachment processes. TIRF microscopy was first described by Axelrod and colleagues in 1983 (Axelrod et al., 1983).
TIRF microscopy captures excitation of a fluorescence marker in the plane immediately adjacent to an interface between two materials having different refractive indices – e.g. a glass coverslip and cell suspension. When light passes between two materials with different refractive indices, refraction or reflection will occur depending on the incident angle. At a particular angle, termed the critical angle, the light is entirely reflected in the plane of the interface. However, when total internal reflection happens, the light energy also generates a weak electromagnetic field (an evanescent wave) that extends into the media with the lower refractive index – in microscopy this is then the cell suspension sample. The magnetic strength of the field decreases exponentially away from the reflection interface, and can only illuminate fluorophores within a few hundred nanometers of the interface. TIRF microscopy provides enhanced close-surface imaging by substantially reducing background fluorescence from cell regions beyond this distance and is therefore optimally suited for the study of cell adhesion. TIRF microscopy has been applied to many cell membrane related studies such as fibroblast cells (Geggier and Phur, 1999; Partridge and Marcantonio, 2006).

Our findings using Alexa-488 wheat germ agglutinin labeled parasites with TIRF methodology are consistent with the ‘suction’ model of attachment mechanism and furthermore suggest that the protrusion of the parasite cytoplasm through the bare zone and unidirectional flow under the ventral disk generated by the ventral pair of flagella may be indications of the negative pressure as a driving force of parasite attachment.

**Materials and methods**
Cell culture

*Giardia lamblia* trophozoites (isolate WB1267) were maintained anaerobically in 8 ml borosilicate glass tubes. *Giardia* parasites remain attached to the walls of these tubes at 37°C. Parasites were grown at 37°C in modified TYI-S-33 media (Keister, 1983), where the phosphate solution was substituted with 0.024 M sodium bicarbonate and 1% (volume) penicillin-streptomycin-amphotericin B was included to prevent culture contamination. For all experiments, *Giardia* cultures were grown to mid-log phase (~80% confluency).

Wheat germ agglutinin cell labeling

For cell labeling, culture media was decanted from culture tubes and 8 ml of fresh culture media supplemented with 10 µg/ml wheat germ agglutinin (WGA) conjugated to Alexa-488 (Invitrogen Inc.) was added for a 2-hour incubation at 37°C. Culture media containing the fluorescence label was decanted, cells were washed three times with 8 ml of modified TYI-S-33 medium at 37°C to remove free fluorescent label, and a final 8 ml of modified TYI-S-33 was added to the tubes. Cells were detached by chilling the tube on ice for 30 minutes, and 400 µL of the labeled culture was loaded onto each well of the 4 center wells of 8-wells Lab-Tek chamber slide (Cat. No. 177445, Nunc Inc.) in which the bottom was covered by clean coverglass (24×50 mm, Fisher Scientific Inc.).

Confocal laser scanning microscopy images

Uniformity of the WGA surface label was confirmed through the use of confocal microscopy. The Lab-Tek chamber slides were placed in the 37°C incubation chamber of
a Zeiss 510LSM/META/NLO live imaging multiphoton microscope confocal laser scanning microscope and viewed with a 488 nm laser source at 60X magnification. Approximately 40 Z-sections at thicknesses of 20 nm were taken and reconstructed in three-dimensions by Zeiss 510 META software.

**TIRF microscopy**

Points of contact between the parasite and the glass coverslip of the Lab-Tek chambers were examined using TIRF microscopy. The Lab-Tek chamber slides were placed in the 37°C incubation chamber of an Olympus IX81 Total Internal Reflection Microscope for observation. As parasites began to attach to the glass coverslip as the media warmed to 37°C, focus level and incident angle of the argon 488nm laser were adjusted to obtain the best TIRF images. Images were taken at 60X using a Plan Apo 60X oil/N.A. 1.45, WD 0.1 mm with correction collar objective, captured using a Hamamatsu C9100-12 EM 512×512 back thinned CCD digital camera driven by IP Lab Suite software with motion control. Wide-field images corresponding to TIRF images were taken as controls to show the full view of cells.

TIRF and wide-field images were analyzed using Metamorph™ software to quantify fluorescence intensity, an indirect measure of distance between cell and substrate assuming uniform cell surface labeling. Background levels were set by calculating the mean pixel intensity from four regions around the cell of interest. Pixel intensities along a line drawn through the cell image were measured and the background subtracted from each reading.
**Fluorescent microsphere experiments**

To examine the flow dynamics and determine the distance between various parasite structures and the substrate, we included fluorescent microspheres in chambers with either labeled or unlabeled *Giardia*. Three types of beads were used: 200 nm diameter carboxylate-modified microspheres with yellow-green fluorescence (505/515), 20 nm diameter carboxylate-modified microspheres with yellow-green fluorescence (505/515), and 20 nm diameter carboxylate-modified microspheres with nile red fluorescence (535/575) (Invitrogen, Inc.).

**Results**

**Surface labeling of *Giardia* using wheat germ agglutinin**

TIRF microscopy requires surface labeling of the cells to be studied. Based on numerous previous studies that successfully used wheat germ agglutinin to label the surface of *Giardia* parasites through its interaction with membrane glycoproteins (Hill et al., 1981; Ward et al., 1988 and Ortega-Barria et al., 1990), we chose to label intact live parasites with WGA conjugated to Alexa-488. Lectins have been shown to block both cell cycle progression and encystation in longer incubations, but exhibit no deleterious effects in the shorter incubations we used here (Ortega-Barria et al., 1994; Meng et al., 1996b).

Recent studies have indicated that parasite surface labeling may be punctate rather than uniform, although no explanation was provided for this observation (Ratner et al., 2008). To examine the uniformity of our WGA labeling, we conducted confocal laser
scanning microscopy followed by three-dimensional reconstruction to visualize the pattern of fluorescence on the cell surface of *Giardia*. This three-dimensional image shows even labeling of ventral surface structures by the fluorescent WGA at lower magnification, with very fine punctate fluorescence revealed at higher magnifications (Supplemental Video 1). These observations partially confirm those of Ratner and colleagues (2008) although the cell surface labeling we observe is significantly more even than what they noted, likely as a consequence of differences in our labeling conditions. Considering the possible important role of ventral disk and anticipating dynamic change of ventral disk topology during attachment and detachment, the even labeling of ventral disk is an especially important precondition for TIRF microscopy, and our WGA labeling meets the necessary criteria.

**Defining the points of contact between *Giardia* cells and the substrate**

TIRF microscopy was used to examine the points of contact between the WGA-labeled parasite plasma membrane of attached *Giardia* parasites and a glass coverslip. In TIRF microscopy, points of contact will appear bright, while more distant portions of the cell surface will be dark. To verify that this is a consequence of the properties of TIRF microscopy, and not the result of uneven surface labeling, we include widefield whole cell images corresponding to the TIRF images.

The TIRF microscopy images document differences in fluorescence intensity for different ventral structures of the parasite (Figure 3.1). The specific pattern shows bright fluorescence for the bare zone of ventral disk and lateral shields, two triangular shaped surfaces posterior to ventral disk and flanking the ventral groove, with slightly fainter but
noticeable fluorescence of the lateral crest. This pattern was consistent across hundreds of cells observed. Based on the principle of TIRF microscopy, the closer the membrane is against interface with the substrate, the stronger the fluorescence signal. So the patterns observed in TIRF microscopy images indicate the most tightly contacting parts of ventral side of attached cells. Previous interference-reflection microscopy images revealed the close contact between the lateral crest and the substrate, while the ventrolateral flange seemed to be in close contact to the substrate (Erlandsen and Feely, 1984); other studies also mentioned that the ventrolateral flange appeared to closely contact substrates (Friend 1966; Sousa et al. 2001; Erlandsen et al., 2004), but after comparing the diameter of the ring shape in our TIRF microscopy images and wide field whole cell images, it is clear that the fluorescence we detect on the anterior portion of cells is due to the lateral crest and not to the ventrolateral flange. The close contact between the bare zone and substrate is a new observation, as is the observation of the lateral shields.

Given the apparent relatively brighter intensity of the fluorescent area of the bare zone compared to the lateral crest, we wanted to determine whether the difference in intensity was the result of different distances between the substrate and membranes or a consequence of the larger patch of plasma membrane contributing to the fluorescence of the bare zone. If the substrate-membrane distances are different, then each pixel should have a different light intensity; if the substrate-membrane distances are the same, then each pixel should have a comparable intensity but more pixels should be bright in the region of the bare zone. We used Metamorph™ software to determine pixel light intensity values corresponding to the lateral crest and the bare zone for images captured
such that the brightness values were in a linear range. These data were calculated for linear transects through 121 TIRF images from 30 attached cells (1 or 2 linear transects for each image), and the results are summarized in Table 1. The mean intensities of lateral crest and bare zone are almost identical, indicating they are approximately equally tight against the substrate surface.

**Dynamic changes on ventral side of attaching and attached cells**

Information about the dynamic changes on the ventral side of cells during the process of attachment and detachment is scarce. Previous studies suggested contractile dynamics of the lateral crest may participate in the attachment or detachment process (Campanati et al., 2002). With TIRF microscopy, we can observe the membrane and structural dynamics of the ventral side of living cells as they attach to and detach from the substrate surface. Representative images of the process can be seen in Figures 3.2 and 3.3, and videos of cells undergoing attachment or detachment are present in supplemental materials. As parasites approach the substrate to begin the attachment process, the lateral crest and lateral shields first become visible (Frames A-E, Figure 3.2). We note, however, that these structures are visible in both cells that are truly initiating attachment and cells that are still moving along the surface of the substrate. Changes to the bare zone are the most notable dynamic phenomenon of *Giardia* cells that are truly attaching; a gradually enlarged bare zone of an attaching cell is observed while establishing attachment (Frames F-J, Figure 3.2). For steadily attached cells, the bare zone, lateral crest and lateral shields are the brightest fluorescent zones in TIRF microscopy images. But for cells beginning detachment and transforming to free swimming status, the bare
zone brightness diminished before the brightness of the lateral crest and lateral shields diminished (Figure 3.3), again signaling that it is the bare zone that signals tight attachment to the substrate.

**Ventral disk of attached cells defines a relative closed space**

The close contact between the lateral crest and the substrate surface is consistent with previous studies (Erlandsen and Feely, 1984). The strong fluorescence signal from lateral crest zones indicates the distance between lateral crest and substrate surface should be quite close to the glass substrate, but directly quantifying this distance is difficult. In order to gain more precise estimates of the distance between parasite and substrate, we added two sizes of microspheres with yellow-green fluorescence (505/515 nm), 200 nm and 20 nm diameter, separately, into medium in which *Giardia* cells were incubated. We chose these two microsphere sizes because their sizes fall within the typical range of the evanescent field.

In TIRF microscopy conditions, only microspheres very close to the surface can be observed, and they appear to move freely in the medium while some of them seemed to stick to glass surface. If the microspheres move close to the surface and then move back away from the substrate where the distance is too long to allow evanescent field exciting fluorescence, the microspheres seem to disappear. When cells are attached to surface, because the thickness of cell exceeds the effective evanescent field, the microspheres are often excluded from the surface under the cells and we typically do not detect microspheres in regions of attached parasites. Thus, in TIRF microscopy performed with unlabeled parasites and labeled microspheres, the parasites appear as dark
areas because of the exclusion of the microspheres. However even when the Giardia cells were labeled by Alexa-WGA, the areas that are attached by cells can be easily distinguished from background because the attached Giardia cells defined darker spots under the parasite away from the bright zones of attachment (Figure 3.4 and Supplemental Video 2).

After further examinations of these images, we noted that the areas corresponding to anterior halves of attached cells were darker than posterior halves (Figure 3.5 and Supplemental Video 3). The anterior half corresponds to ventral disk area while the posterior half of Giardia is mostly ventral groove where ventral flagella emerge posterior to the ventral disk. Because darker regions indicate a reduction in the microspheres under the ventral side, this observation points to a barrier between the space under ventral disk and ambient space. The difference in shades of darkness can be explained by the accessibility difference of the space beneath ventral side. Occasionally, we directly observed moving microspheres of 20 nm get close to lateral crest but could not move under it. Collectively, these observations indicate that the distance between ventral lateral and substrate surface is less than 20 nm.

We occasionally also observed microspheres enter into the half-closed ventral groove space. This phenomenon is especially clear for cells in medium with 200 nm microspheres. The microspheres in the ventral groove space do not move as freely as they do away from the parasites, and their movement seems to be correlated to the beating of ventral flagella (Figure 3.6 and Supplemental Video 4).

**Flow in the marginal groove formed by lateral crest and ventrolateral flange**

85
A previous study by Ghosh et al. reported on the movement of beads under the ventral surface of attached parasites (Ghosh et al., 2001). They suggested the possibility that the beating of the ventral flagella might cause this flow and possibly act as a means of moving nutrient-rich media around the parasite for feeding. In this study we sought to extend these findings by further characterizing the movement and the parameters of the space and flow within the marginal groove.

We were able to observe microspheres travel around the periphery of the anterior end of the parasite in a well-defined arc of movement, as if the beads were trapped in a circular trail. This phenomenon was more readily observed when cells are in medium with 20 nm beads. Interestingly, however, we did not observe the unilateral direction of movement implied by Ghosh and colleagues (their data is not shown, so we are limited to their interpretation of the data in our comparison). Instead, the microspheres move along a curve with a back and forth, tick-tock, motion (Supplemental Video 2). One interpretation is that the beads are indeed trapped within the marginal groove. The beating of ventral flagella results in the change of direction of fluid movement so that the microspheres trapped in the groove also move back and forth. The difference of 200 nm and 20 nm microspheres suggests either that the diameter of this groove may not be big enough for 200 nm microspheres or that the 200 nm microspheres cannot move along the channel freely.

To directly confirm that the beads were indeed moving within the marginal groove, we sought to better define the relative location of the microspheres to cell structures. Because the fluorescent signal from microspheres with yellow-green
fluorescence (505/515) is much stronger than the signal from the fluorescent WGA which labels the cell surface, we cannot observe microspheres and cell at the same time under TIRF conditions. We instead used 20 nm microspheres labeled with Nile red, which excites a similar intensity of fluorescence as the fluorescent WGA moieties on the cell membrane in these double-labeling studies. The TIRF microscopy videos revealed that the beads indeed moved along the marginal groove between lateral crest and ventrolateral flange (Supplemental Video 5).

We observe that only a subset of attached cells exhibited this kind of circular bead movement pattern. This could be explained by one of two possibilities: either access to the marginal groove is restricted such that most microspheres cannot gain entry or that the ventrolateral flange in most cells is not closely apposed to the substrate such that most beads that enter this space simply move out again. Because we do not observe the ventrolateral flange well in most attached cells under TIRF conditions, we believe that the ventrolateral flange is not tight against the substrate and that most beads that enter this space will simply not be trapped. Occasionally, the trapped beads in the marginal groove clump together. Even after cell detachment, the clumped beads remain attached to the substrate surface and can be seen as a fluorescent print (Figure 3.7 and Supplemental Video 6).

**Beads entering and exiting ventral disk travel along the route corresponding to the spiral form of the ventral disk**

much to our surprise, we occasionally noted an important phenomenon in our microsphere experiments: 20 nm but not 200 nm microspheres moving under the ventral
disk in an anterior to the posterior direction. After repeated observations of this phenomenon, we identified the exact point where microspheres enter into the ventral disk space and noted that it was a single, precise zone at the anterior end of the disk, slightly offset from center. The beads then begin along a defined path toward the posterior end of the disk, moving in a clockwise (from ventral side view) arc before gaining more freedom of movement and exiting out the posterior end of the disk into the ventral groove. Strikingly, this pattern follows the spiral structure of the ventral disk. The surface of the ventral disk has a partial overlap of its structure along the spiral line of its microtubule base (Holberton, 1973a, b). This spiral shape is sufficiently well-defined to be visible in the footprints left on the surface of epithelial cells following parasite detachment (Erlandsen and Chase, 1974; Owen et al., 1979; Chávez et al., 1986; Sousa et al., 2001). The point where the microspheres enter is where the ventral disk surface overlaps at the very anterior end of the cell (Figure 3.8 and Figure 3.9) (Supplemental Video 7). This region exhibits a small gap formed by overlapping surface of the spiral array of microtubules as noted in numerous scanning electronic microscopy images (Holberton, 1973a, b; and Elmendorf et al., 2003). This gap may allow a microsphere to align directly with it to enter the relatively closed space beneath the ventral disk. For the microspheres that have moved into ventral disk space, they are restricted in the ventral disk space for a short period of time. They sometimes moved close to the lateral crest from the inside of disk space but cannot pass underneath the crest, which supports our earlier observation that the lateral crest is in close contact with substrate surface (Supplemental Video 8). The beads often lingered under the disk for a few moments
before being expelled at the posterior end of disk where the overlap of the spiral again results in a more significant gap that has often been noted in electron micrographs (Holberton, 1973a, b; and Elmendorf et al., 2003) (Figure 3.9 and Supplemental Video 9). Importantly, the direction of flow is always anterior to posterior, indicating the outside pressure is greater than the pressure under the ventral disk space, and that there is a unidirectional flow created under the disk.

**Discussion**

The virulence of *Giardia lamblia* relies on its ability to attach to host epithelial cells of the small intestine. But the exact method by which *Giardia* achieves this attachment is not clearly understood. Because *Giardia* can attach to biological and inert substrates (Knaippe, 1990), and recent research has demonstrated that the attachment force is insensitive to surface treatments (Hansen et al., 2006), the basic attachment force is more likely to be a process involving a dynamic biomechanical mechanism. Thus, while it seems likely that the lectin attachment model may play a role in *Giardia* attachment to host intestinal cells *in vivo* – perhaps as a means to initiate contact between the parasite and host intestinal mucosal surface independent of parasite orientation – the dominant mechanism mediating attachment must be independent of specific molecular or charge interactions. The ‘clutching’ (grasping of the substrate by the lateral crest of the ventral disk) or ‘suction’ (generation of negative pressure under the ventral disk of the parasite) models of attachment are the most likely candidates. Resolution between these two models requires more detailed understanding of the dynamics of the attachment
process, in particular a delineation of the interaction and juxtaposition of the various structures on the ventral side of the parasite and the substrate. Here we have used TIRF microscopy with fluorescently-labeled parasites and fluorescently-labeled microspheres to better evaluate the proposed models or provide new mechanisms of attachment.

We have observed that the lateral crest, bare zone and lateral shields are the zones on the ventral side of the parasite that contact the substrate surface most closely in attached parasites. The beating ventral flagella were also observed under TIRF conditions when glimpses of the undulating flagella beat close to the surface, reinforcing the power of the TIRF technique. The fluorescence signals corresponding to lateral crest and bare zone of attached cells are very similar based on measurement results of Metamorph™ software suggesting the contact surface between the ventral crest to the substrate surface, and the bare zone to the substrate surface, are at the same level. Our findings confirm the close contact between the lateral crest and substrate surface which has been noted previously by other researchers. However, our observation of the close contact between the bare zone and surface and between the lateral shields and surface are new and important findings.

We also capture for the first time the changes in the the parasite-substrate contact points during the attachment and detachment process and find that the prominence of the bare zone is the primary indicator of attachment: in attaching cells, the bare zone increases in size and proximity to the substrate, while in detaching cells, the bare zone slowly recedes. The bare zone is the region at the center of the ventral disk – a hole in the disk formed by the spiraling of the microtubules around a midpoint and therefore
cleared of cytoskeleton structures. Thus, we interpret the increased prominence of the bare zone in attaching cells as a protrusion of the flexible cell body through this hole in the disk to form a pedestal under the disk. This protrusion requires a force for its creation. The force could either take the form of a positive pressure exerted on the cell dorsal to the ventral disk to force the cell body through the disk or it could be a negative pressure under the disk that would draw the cell body into the space. Resolution between these two options and identification of the source of the pressure will require further research.

Incorporation of fluorescent microspheres also allowed us to more accurately quantify the distance between cell surface and substrate. We observed that it was exceedingly rare for even the 20 nm microspheres to move under the ventral disk of attached parasites – a finding that contradicts the undocumented report by Ghosh and colleagues (2001) that mentioned that beads readily moved from the external environment to the space under the ventral disk. We cannot explain this discrepancy but the power of resolution in the plane of attachment afforded us by TIRF microscopy makes us confident of our findings.

Incorporation of fluorescent microspheres into the TIRF microscopy also allowed us to investigate the nature of fluid flow around attached parasites. In our findings we first demonstrated the possibility that ventral disk and ventrolateral flange can form a marginal groove – in effect a tunnel between the two structures and substrate. In a subset of cells the microspheres can be trapped in this groove and move along the space providing by the gap. Notably in most cells we did not observe these trapped
microspheres in the marginal groove, a finding that corroborates our TIRF imaging of contact points to indicate that the ventrolateral flange is often not tightly apposed to the substrate. Interestingly, the fluid in the gap is moving but not simple clockwise or counter-clockwise, but rather back and forth causing the microspheres to move in a tick-tock motion. While we do not have a definitive cause for this fluid flow, we suggest that the beating of ventral flagella may create turbulence in the marginal groove resulting in shifting directions of flow. The size of the marginal groove appears to be on the order of <200 nm diameter based on the relative ease with which the 20 nm microspheres, but not the 200 nm microspheres, could move in this space.

One striking finding was our observation of the relatively rare instances when a microsphere entered the space under the ventral disk. Most of the time, beads within the marginal groove move without entering into the disk space, but when they do, they follow a specific route. This route initiates at the point where the overlapping line of the ventral disk surface meets the outline of the ventral disk, a region just offset from the midline at the anterior end of the parasite; this location again contradicts the report from Ghosh et al. who noted entry at the “side of the parasite”. We do not know if the rarity of this event indicates the relative size of the gap at the anterior end of the disk or a very low flow rate to draw the microspheres in. Three features distinguish this microsphere motion under the ventral disk from the movement of microspheres in the marginal groove: (1) Unlike microspheres that move in the marginal groove both back and forth, the entering microspheres always move directionally from the anterior end to under the disk before expulsion our the posterior end of the disk; (2) The microspheres under the
ventral disk move faster than those within the marginal groove; (3) While under the disk, the microspheres move only in a clockwise direction, loosely tracking in the space between the edge of the bare zone, the ventral disk spiral and the lateral crest. The unidirectional tracking of the microspheres under the ventral disk indicates that the pressure inside the disk space is lower than the external environment at the anterior end of the parasite and higher than the pressure in the ventral groove at the posterior end of the disk. This finding provides direct support for negative pressure based mechanism.

We propose the following model of attachment based on our TIRF microscopy. When establishing attachment, the bare zone area on ventral disk surface protrudes. We currently favor a mechanism in which negative pressure under ventral surface of the parasite generates the force necessary for this dynamic. The continued beating of the ventral flagella that has been observed by numerous researchers will expel fluid from the ventral groove, which is half-closed because lateral shields on each side of groove are closely contacted substrate surface as well as lateral crest. This expulsion of fluid will, in turn, generate additional negative pressure in the ventral groove further drawing fluid from under the ventral disk and marginal groove and thereby serving as a continual source of negative pressure under the disk. Generally, our hypothesis is that attachment is maintained via the structures on ventral side of attached cells providing relatively closed space to maintain negative pressure as well as the negative pressure being generated by flagella beating. Investigation of the negative pressure in more detail (e.g. estimation of the force provided by negative pressure via measurement of flow speed and volume...
underneath attached cell) should be next steps to better understand the attachment mechanism.
Figures and table

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The average ratio of lateral crest over bare zone: $1.002\pm0.04$

Table 1: Summary of measured gray intensity of lateral crest and bare zone. ‘*’: Lateral crest > Bare zone means the gray intensity value of lateral crest is larger than the gray intensity value of bare zone. The average ratio of gray intensity between lateral crest and bare zone suggests similar distance from lateral crest or bare zone to the substrate surface of attached cells.
Figure 3.1: The bare zone, lateral crest and lateral shields are most significant areas on ventral side of attached cell against substrate surface. Both TIRF image (left) and wide field image (right) of same cells attached to glass substrate were shown and compared. Red arrow: pointing to area corresponding to bare zone. Blue arrow: pointing to area corresponding to lateral crest. Yellow arrow: pointing to area corresponding to lateral shields.
Figure 3.2: The process of *Giardia* attachment indicated by consecutive images (with about 0.1 second interval). The red box marked a *Giardia* cell establishing attachment. From panel A to J, the marked cell began and finally established attachment. The edge of
disk and lateral shields contacted the substrate surface first then the bare zone gradually contacted the surface.
Figure 3.3: The process of *Giardia* detachment indicated by consecutive images (with about 0.1 second interval). The red arrow marks the *Giardia* cell began to detach. From panel a to h, bare zone attachment diminished during detachment process and bare zone detached faster than lateral shields.
Figure 3.4: The dark spot formed by attached *Giardia* cells. In panel A, the cells are untreated while in panel B the cells are labeled with Alexa-488 conjugated WGA. In both panels, left image is taken under TIRF condition and the right image is wide field. Red arrow points to attached *Giardia* cell.
Figure 3.5: The anterior half is darker than the posterior half of the dark spot formed by attached *Giardia* cells. Left image is taken under TIRF conditions and the right image is a wide field image. The red arrow points to anterior half of attached *Giardia* cell. The yellow arrow points to the posterior half of an attached *Giardia* cell.
Figure 3.6: Four consecutive images (frames a to d with about 0.1 second interval) showing the 200 nm beads underneath the ventral groove area of an attached *Giardia* cell (in red box).
Figure 3.7: Beads print formed by 20 nm beads left by first attached and then detached *Giardia* cell. There are four consecutive images from same video (with about 0.1 second interval): a. attached cell, b. cell began to move, c. cell move further and d. a clear print left. The red arrows point to the bead print and the yellow arrows point to the moving cell.
Figure 3.8: The TIRF image showing the ventral side of *Giardia*. The yellow arrow points to ventro-lateral flange. The red arrow points to ventral disk. The spiral shape of ventral disk surface is clearly show and the ventral disk surface overlaps along the spiral line.
Figure 3.9: The process of a bead entering the space between ventral disk of attached *Giardia* cell and substrate surface. a to f are consecutive images (with about 0.1 second interval) from same video showing the bead movement during this process.
CHAPTER 4

Conclusion and Discussion
4.1 Conclusion

The work in this dissertation makes contributions to our understanding of both molecular and mechanistic aspects of Giardia attachment: (1) the first time microfilament-associated proteins in Giardia have been identified and (2) direct support for a negative pressure model of attachment from the observation of the points of contact between parasite and substrate in attached and attaching/detaching cells and of parasite-induced flow dynamics by TIRF microscopy.

The research described in Chapter 2 identified α-1 and α-7.3 giardins as the first known microfilament-associated proteins in Giardia and extend our understanding of annexins as microfilament-associated proteins. The detail description of attachment and detachment dynamics in living cells described in Chapter 3 provides more accurate information about the contact zones and change of surface topology via edge-cutting TIRF microscopy. In addition, further study with fluorescent microspheres reveals the presence of flow underneath the ventral disk of attached cells. Collectively these TIRF microscopy studies provide evidence consistent with a negative-pressure based attachment mechanism.

4.2 Discussion

4.2.1 Identification of α-1 and α-7.3 giardins as microfilament-associated proteins

Influence of actin in co-sedimentation experiment

In our study, human β-actin was used instead of Giardia actin based on the similarity of sequence level and predicted structural conservation of Giardia actin to
human β-actin as described in Chapters 1 and 2. For the co-sedimentation assay, large amounts of high quality actin were needed. Our lab successfully expressed recombinant Giardia actin in E. coli and succeeded in making large quantities for polyclonal antibody production, but actin requires chaperones to fold properly – chaperones that are not present in E. coli. We chose not to perform these assays using a protein of undetermined structure and instead tried to express Giardia actin in a baculovirus/Sf9 insect cell protein expression system but failed to obtain sufficient quantities. Although extracting actin from Giardia cells may be a possible way to collect Giardia actin directly, the quantities required would be a great challenge for our tissue culture system; in mammalian cells, actin is one of the most abundant proteins, but it is a relatively non-abundant protein in Giardia. This point was also verified by analyzing our Giardia lysate sample for co-sedimentation experiments. Thus commercially-available human β-actin was chosen as a substitute for Giardia actin. Structural homology modeling predicts a very high degree of structural conservation, particularly along the faces of the protein known to bind to several microfilament-associated proteins. Despite this, it is possible that some Giardia proteins will be components of the Giardia microfilament system but will be unable to bind to human β-actin due to subtle differences in binding sites between the actins. Thus, our actin co-sedimentation assay is likely to yield an incomplete list of microfilament-associated proteins in Giardia. However, before this research, not a single microfilament-associated protein had been identified in Giardia, thus identification of any protein as microfilament-associated protein is a large leap forward, and this work will
contribute to further identification of complete microfilament-associated proteins in *Giardia* in the future.

**Influence of the lysate in co-sedimentation experiment**

*Giardia* lysate is the second critical component of the co-sedimentation assay. In our study, we prepared a lysate composed of only soluble cytosolic proteins from *Giardia*. This procedure was designed to remove nuclei and other large cytoskeletal structures from the lysate to minimize contamination of co-sedimentation fractions with irrelevant aggregates of proteins. In order to release potential microfilament-associated proteins from cytoskeleton system into the soluble cytosolic fraction, cytochalasin-D was also added to lysis buffer at a concentration of 10 µM, in accordance with findings from experiments using cytochalasin-D to disrupt attachment and alter actin localization by immunofluorescence microscopy. This drug was removed to prevent its interference in the actin co-sedimentation assays.

After the co-sedimentation experiments, the composition of lysates was analyzed by mass spectrometry. Some typical cytosolic proteins, such as arginine deiminase, BiP, and ornithine carbamoyltransferase, are very abundant, consistent with serial analysis of gene expression (SAGE) and mass spectrometry (MS) data on the *Giardia* genome database site. But α giardins were actually less abundant in our soluble cytosolic fractions compared to the genome SAGE and MS data. This is reasonable because α giardins are usually distributed around plasma membrane and may interact with cytoskeleton system, which would have reduced their abundance in the lysate. Importantly, this bias provides
further support for our argument that the enrichment of selected α giardins in actin co-sedimentation assays is specific.

Identification of microfilament-associated proteins in Giardia

Our results from the actin co-sedimentation assay revealed that only Giardia actin, α-1 giardin and α-7.3 giardin were significantly enriched in human β-actin pellet comparing to control pellets (P<0.2). Several members of the highly related α giardin family appear to be among the very few other proteins to be selectively enriched in actin co-sedimentation pellets. Beyond α-1 and α-7.3 giardin, α-2 giardin and α-6 giardin are enriched, although with a p value below our cut-off for significance. The known association of annexins with actin in other eukaryotes (reviewed by Hayes et al., 2004) and the observed co-localization of actin and α-1 and α 7.3 giardin at the parasite plasma membrane (Weiland et al., 2005) provide further indirect support for a functional role in vivo for this observed interaction. The interaction between α giardins and actin not only indicates the specificity of co-sedimentation method, but also suggests the possibility that the failure of α-2 giardin and α-6 giardin in present study to meet statistical significance may result from the use of human β-actin as an imperfect substitute. Further studies should use Giardia actin and pay close attention to other α giardins, not limited to α-1 and α-7.3 giardin.

4.2.2 TIRFM observation and analysis of Giardia attachment

Fluorescence labeling
The proper labeling for materials is crucial in fluorescence microscopy. We labeled the *Giardia* trophozoites surface with wheat germ agglutinin (WGA) conjugated with Alexa-488. Wheat germ agglutinin can specifically bind to lectins distributed on the surface of trophozoites (Ward et al., 1988). Usually TIRF microscopy is used to observe the adhesion of mammalian cells, like fibroblasts, to a substrate. The dimension of *Giardia* is much smaller than fibroblasts, while the structures on the ventral side are very complicated compared to the relatively uniform surface of many undifferentiated mammalian cells providing an interesting challenge for our research. Especially when we consider possible contact zones, for example the lateral crest, which is the outer rim of the ventral disk, the contact surface will be relatively tiny compared to parasite dimension. Recent experiments have shown that surface labeling with WGA can be spotty at a high level of resolution, perhaps reflecting an uneven lectin distribution (Ratner et al., 2008) -although our labeling conditions appear to have attained a more even distribution of label. This likely explains why usually the fluorescent signal from lateral crest is not as even as the bare zone which has a larger surface area. One alternative to our current approach would have been to use a fluorescent marker to label only the liquid phase and not the parasite. This technique is called total internal reflection aqueous fluorescence (TIRAF) microscopy. By combining TIRF and TIRAF microscopy, the observations could complement each other to help provide a more thorough recording of dynamic changes on ventral surface.

*Estimate the distance between cell and substrate*
In this work we also used fluorescence microspheres to estimate the distance between the parasite and substrate surface. This is an admittedly rudimentary estimate limited by the size of microsphere that are available, but it provides us with the first such measurements ever taken for live *Giardia* – all previous data coming from electron microscopy studies. In fact, TIRF microscopy technique can also be used to calculate the distance between cell membrane and substrate. As mentioned in Chapter 1, the intensities of fluorescence signal can be expressed as the function of the index ratio, the incident angle and the distance between a specific location on membrane to the substrate surface. Due to the capacity of the TIRF microscope used in this research, we cannot record the incident angle of the laser. If we do and measure the maximum intensity of fluorescence marker by marker attached directly to interface surface, the distance between the contact zone and substrate surface can be calculated. Our current techniques limit us to measuring only relative distances between parasite surfaces and the substrate.

4.3 Future directions

The studies presented in this dissertation are meaningful for deepening our understanding of *Giardia* cellular biology, *Giardia* attachment dynamics, and furthering our understanding of the evolution of eukaryotic cytoskeleton. But these are just the initial steps for many important areas for *Giardia* research. In this part we discuss the potential future directions, both molecular and mechanism levels, based on our present findings.

4.3.1 Protein research
The localization and function of proteins

As described in Weiland’s paper examining the α giardins (Weiland, et al., 2005), AU-1 epitope tagging of the α-giardins was used to localize α giardins. Of the 21 α giardins, 13 were localized: localization of 4 proteins was not attempted in these studies, and overexpression of epitope-tagged constructs for 4 other proteins proved lethal to the parasites. Interestingly, the α-1 and α-7.3 giardins identified in our study are localized to plasma membranes, where actin is also present. Despite the information about α giardins’ localization, the functions of α giardins in Giardia remain unclear. Our best insights come from their identification as annexin homologues. However, the diversity of annexin functions prevents a simple functional extrapolation.

In present study, we identified α-1 and α-7.3 giardins as microfilament-associated proteins. Because gene knock-out experiments are not feasible in Giardia due to the tetraploid nature of the parasite, a functional investigation of α giardins can be carried out through antisense hammerhead ribozyme knock-down experiments to decrease the expression level of select α giardins (Dan et al., 2000). The morphology and attachment ability of modified Giardia could then be observed to determine the possible function of these proteins in microfilament behavior.

Protein-protein interaction

Besides knockdown experiments to reduce the expression level of specific α giardins and subsequent analysis of effect on cell functions, protein-protein interaction detection experiments can either verify the interaction between α-1 and α-7.3 giardins to actin or detect possible interaction of these giardins to other proteins or structures in
living cells. For example, the interaction between α-1 and α-7.3 giardins to actin can be verified by co-sedimentation or immunoprecipitation assay. The interaction of giardins to other proteins can be detected by fluorescence resonance energy transfer (FRET) techniques or Biacore system using surface plasmon resonance (SPR) technique. The SPR technique is particularly powerful for identification of novel binding partners and can provide information such as specificity, affinity, kinetics and binding partner. FRET allows only the \textit{in vivo} testing of two proteins already presumed to interact.

\textit{How α giardins regulate the microfilament system}

The influence of α-1 and α-7.3 giardins on actin polymerization can be tested via \textit{in vitro} assays, determining polymerization speed, polymerization percentage, and length of F-actin. Similar studies with \textit{Toxoplasma} and \textit{Leishmania} actins have proven particularly revealing to document the novel dynamics of microfilaments in these other protozoan parasites (Sahoo et al., 2006; Kapoor et al., 2008). \textit{In vivo}, the effect of α giardins on the localization of actin can be detected in living cells by GFP labeling following changes to the expression level of giardins following knockdowns and overexpression experiments. Also these tests can be combined with with drug treatment to check if the behavior of \textit{Giardia} attachment is different in α giardin deficient parasites from control cells. Additionally, the participation of actin on \textit{Giardia} growth and encystation was recently reported by Castillo-Romero et al. (Castillo-Romero et al., 2009). Modifications to actin distribution were observed during encystation. Thus, the role of α giardins in encystation should be investigated by microscopy and drug treatment as well.
Identify the proteome of Giardia microfilament zones

The co-sedimentation method in this proposal cannot identify all microfilament associated proteins in Giardia because some of them are not abundant enough for detection by mass spectrometry or because of the shortcomings of our use of human β-actin as a substitute for parasite actin. Because actin in Giardia is tightly connected to the periphery at the plasma membrane and is associated with the anterior flagella axonemes, we can alternatively isolate these two subcellular fractions and analyze the protein populations in these samples by tandem mass spectrometry assay. Then we can further verify any identified protein’s relation to actin system through the assays described above.

Drug research based on the identified microfilament associated proteins

An important aspect of Giardia research is novel drug development for treating giardiasis. With the identification of microfilament-associated proteins, more potential drug targets will be available based on the structural differences between these Giardia microfilament-associated proteins and mammalian microfilament-associated proteins. The difference between α giardins and human annexins are more significant than the difference between Giardia actin and human actin. Thus the drug that can interrupt the normal function of α giardins may have less of an effect on host annexins and display reduced toxicity in pharmaceutical use.

4.3.2 Microscopy research
**Microsphere experiment**

Our data are consistent with a negative pressure-based model. But the mechanism for generating negative pressure is still not clear. The data currently suggest that the negative pressure is generated by the ventral flagella beating. Thus, the further study of the movement of beads underneath ventral side and modeling of fluid flow dynamics will be necessary to provide clues to finally clarify the method for generating of negative pressure.

**Flow force, tonic shock and surface modification**

Forces created by flow and tonic shock are two factors that can cause detachment of already attached cells (Hansen and Fletcher, 2008). Now that we have established the technique to observe *Giardia* attachment via TIRF microscopy, the combination of TIRF microscopy with these variables as treatments of attached parasites should reveal the dynamic changes happening during detachment or even modification to contact zone of attached cells before detachment. Alternatively, although the surface treatments do not change attachment force of attached *Giardia* cells (Hansen et al., 2006), the patterns of contact zone that contact against substrate surface most closely under different surface conditions may be different. The cover slide that encloses the chamber used in TIRF microscopy experiment can be modified to either positive or negative charge and to either hydrophilic or hydrophobic surfaces. Similarly, fluorescent microsphere with different surface characteristics can also be used to test the influence of surface modification to bead behavior. These extensions of our current experiments should provide even more
detail regarding the mechanism and dynamics of the biomechanical processes that regulate attachment.

4.4 Summary

The work described in this dissertation investigates the molecular and mechanistic basis of Giardia attachment. In Chapter 2, studies focused on molecular interactions, and α-1 and α-7.3 were identified as microfilament-associated proteins by co-sedimentation method; this work extends our knowledge about the Giardia microfilament system beyond Giardia actin alone. In Chapter 3, observations based on TIRF microscopy allowed for a more precise visualization of the plane of attachment than has previously been possible, and our findings provide intriguing insights into the biomechanics of attachment, demonstrating the closest contact of the bare zone, the lateral crest and the lateral shields to substrate surface in attachment and dramatic change in morphology of bare zone during attachment and detachment; this study provides more supportive data for negative pressure-based attachment mechanism. Both directions of this dissertation provide more potential areas that deserve further investigation to better understand questions such as how the microfilament system in Giardia is regulated, what is its influence in attachment, and what is the source of negative pressure? Ultimately the work presented here will not only improve our understanding of Giardia biology, but also contribute to develop drugs against giardiasis via interruption of attachment.


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148
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