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The Role of Actin Depolymerizing Factor in Regulating Actin Dynamics in Toxoplasma gondii

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THE ROLE OF ACTIN DEPOLYMERIZING FACTOR IN REGULATING ACTIN DYNAMICS IN *TOXOPLASMA GONDII*

by

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ABSTRACT OF THE DISSERTATION

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in Regulating Actin Dynamics in *Toxoplasma gondii*

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Apicomplexan parasites utilize a unique process of rapid motility termed gliding, which is coupled to their invasion of host cells. Gliding and invasion are dependent on parasite actin filaments, yet parasite actin is mostly non-filamentous. Filaments have been detected only transiently during gliding, suggesting that parasite actin filaments are rapidly assembled and disassembled during gliding motility. Little is known about what regulates the turnover of parasite actin filaments.

In higher eukaryotes the Actin Depolymerizing Factor (ADF)/Cofilin proteins are essential regulators of actin filament turnover. ADF is one of the few actin binding proteins conserved in apicomplexan parasites. To investigate the role of ADF in regulating actin dynamics in apicomplexan parasites, *Toxoplasma gondii* was used as a model apicomplexan, and the activities of *T. gondii* ADF (TgADF) were analyzed *in vitro* and *in vivo*. We found that TgADF engaged in dual activities. In contrast to most

ADF / Cofilin proteins, TgADF was found to be a potent actin monomer sequestering protein that strongly inhibited actin polymerization, suggesting that it likely functions to maintain high G-actin concentrations in the cytoplasm of non-motile parasites. This role was reflected in its molecular structure, in which conserved G-actin binding sites were maintained, while key F-actin binding residues were absent. Despite this, TgADF demonstrated the ability to promote actin filament disassembly via a severing mechanism.

Using a conditional knockout system we examined the function of TgADF in the parasite. TgADF was found to be essential for controlling productive gliding motility, and its absence lead to defects in host cell invasion, parasite egress, and parasite dispersal. Detailed analysis of motility revealed that parasites were unable to engage in sustained helical gliding, and moved at markedly reduced speeds. These defects are predicted to arise from the presence of more stable actin filaments in the parasite.

Overall both the monomer sequestering and filament severing activities of TgADF are predicted to serve important functions *in vivo* for maintaining high G-actin concentrations for rapid filament assembly, and disassembling actin filaments for rapid filament turnover, respectively. These studies demonstrated that ADF is essential for regulating actin dynamics in *T. gondii*.

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Chapter 1

Introduction

This chapter was composed entirely by Simren Mehta. Comments from L. David Sibley were incorporated into the final version presented here.

Taxonomy

The phylum Apicomplexa represents a diverse group of organisms consisting of more than 5000 species of mostly parasitic protozoa (Levine, 1988; Gajadhar *et al.*, 1991; Escalante and Ayala, 1994). The defining feature of this phylum is the presence of a group of specific cytoskeletal features and secretory organelles at the apical end of the organism, called the apical complex, which is visible by electron microscopy (Dubey *et al.*, 1998). These features play critical roles in parasite attachment and invasion of host cells, which are essential steps in the parasitic life cycle. A variety of human and animal pathogens are represented in the Apicomplexa, such as *Plasmodium* spp., the etiological agent of malaria, *Toxoplasma gondii*, the causative agent of toxoplasmosis, *Cryptosporidium* spp., the agent of the water-borne disease cryptosporidiosis, and *Eimeria* spp., the causative agent of coccidiosis, a significant economic burden in the poultry business. Interestingly, in contrast to the diversity of species found in other members of the phylum, *Toxoplasma gondii* is the sole species of the genus *Toxoplasma*, and displays limited genetic diversity. Based on the analysis of strains in North America and Europe, *T. gondii* is mainly comprised of 3 clonal lineages, Types I, II and III (Dardé *et al.*, 1992; Sibley and Boothroyd, 1992; Howe and Sibley, 1995).

Life cycle

The *T. gondii* life cycle consists of two independent phases: a sexual stage which occurs in the single definitive host, members of the cat family *Felidae*, and an asexual phase

which, in sharp contrast, can take place in virtually all warm-blooded vertebrates (Dubey, 1977). The apparent lack of need for the sexual stage, combined with the ability to infect all nucleated cells, likely accounts for the widespread prevalence of this successful pathogen.

Sexual cycle

The sexual cycle of *T. gondii* takes place exclusively in the feline intestine (Dubey, 2009). Cats become infected after eating infected animals that have tissue cysts containing the slow growing bradyzoite form of the parasite, or more infrequently from oocysts that have been shed into the environment. Within the cat's stomach and intestine the cyst wall is proteolytically dissolved, releasing parasites that infect the epithelial cells of the small intestine, where they undergo multiple cycles of replication. Following the production of micro- and macrogametes in the intestine, and their subsequent fertilization, a wall is formed around the zygote and oocysts are excreted in the cat feces. At ambient temperatures in the environment, oocysts sporulate giving rise to the highly infectious sporozoite stage parasites, which remain protected in the environment within the highly resistant casing of the oocyst until ingestion by another susceptible host organism (Dubey, 2009).

Asexual cycle

A wide-range of birds and mammals may ingest oocysts from the environment, and become intermediate hosts in the asexual cycle (Dubey, 2009). Within the gut of the animal, sporozoites are released from the oocyst, and subsequently infect the intestinal

epithelium where they differentiate into the fast growing tachyzoite form of the parasite. Dissemination of tachyzoites throughout the body results in acute infection. In response to host innate and adaptive immune pressures, tachyzoites differentiate into the slow growing bradyzoite stage of the parasite, that resides in tissue cysts primarily found in the central nervous system and muscle tissue. This represents the chronic phase of infection. Completion of the asexual cycle occurs when tissue cysts are consumed by another host organism, and rupture of cysts in the gut releases bradyzoites that infect the intestinal epithelium and differentiate back into the rapidly dividing tachyzoite form.

In the laboratory, tachyzoites are propagated via the asexual cycle in cell culture. Tachyzoites are approximately 5 μm long and 2 μm wide and replicate every 6 – 8 hours in a non-fusogenic vacuole via the process of endodyogeny (Dubey *et al.*, 1998). Host cells typically rupture after vacuoles reach a size of 64 - 128 parasites releasing parasites that undergo further rounds of successive replication.

Oral transmission

In North America and Europe, there is very little genetic diversity amongst the *T. gondii* strains found in nature, with strains conforming to one of three widespread genotypes named Types I, II and III (Dardé *et al.*, 1992; Sibley and Boothroyd, 1992; Howe and Sibley, 1995). The absence of genomewide polymorphisms within the lineages suggests a recent genetic bottleneck. The ability to bypass the need for the sexual cycle in the cat seems to be a relatively recently acquired trait that, in North America and Europe, is associated with the 3 clonal lineages, and less so with the exotic strains (defined as those not included under the 3 major lineages, and representing < 1% of infections in North

America and Europe) (Su *et al.*, 2003). It is thought that the acquisition of oral infectivity of tissue cysts has led to the clonal expansion of distinct lineages (Su *et al.*, 2003), although recent work indicates that certain exotic strains in South America that are nonclonal can also produce orally infectious tissue cysts (Khan *et al.*, 2007). Recently it was identified that the highly prevalent *T. gondii* lineages shared a monomorphic version of a specific chromosome (Ia), suggesting that fixing of this chromosome in the population is associated with the success of these lineages (Khan *et al.*, 2007). However the predicted selective advantage conferred by genes on this chromosome has not yet been identified.

Pathogenesis

Humans are accidental hosts for *T. gondii*. Infection occurs via the ingestion of tissue cysts in undercooked infected meat, or oocysts in food and water contaminated with cat feces (Mead *et al.*, 1999). Seroprevalence rates of *T. gondii* are approximately 11% in humans in the US, and up to 80% in other parts of the world such as France and Brazil, where the high consumption of undercooked meat and the large presence of stray cats respectively, contribute to high infection levels (Bahia-Oliveira *et al.*, 2003; Coelho *et al.*, 2003; Jones *et al.*, 2007). After ingestion, parasites cross the intestinal epithelial barrier and disseminate throughout the body via the blood or lymphatics, transforming into cysts in the peripheral tissues.

While infection with *T. gondii* is typically benign in healthy individuals, with at most mild flu-like symptoms, the reactivation of bradyzoites in tissue cysts can lead to

severe complications in immunocompromised individuals, particularly AIDS patients and those receiving immunosuppressive therapy. In AIDS patients, intracerebral focal lesions often result in toxoplasmic encephalitis, which can be fatal if left untreated (Joynson and Wreghitt, 2001). Other manifestations that occasionally occur with immunosuppression include retinochoroiditis, pneumonitis, myocarditis and myositis. Congenital infection, either through a new infection with *T. gondii* or re-activation of a chronic infection in the pregnant mother, is also of concern and can result in miscarriages or stillbirths (Remington and Klein, 2001). Originally occular toxoplasmosis was thought to be primarily associated with the late manifestation of congenital *T. gondii* infection, but is increasingly being associated with acute infections (Holland, 2003).

Standard treatment for *T. gondii* infection utilizes a combination of pyrimethamine and sulfadiazine, although pyrimethamine and clindamycin can also be used (Joynson and Wreghitt, 2001). However these drugs are toxic and only effective against the tachyzoite stage, not the chronic bradyzoite stage. Thus there is a need for less toxic drugs that can eliminate the infection.

Parasite dissemination *in vivo*

After entering the host, parasites must migrate from the initial site of infection to deeper tissues where they cause disease. From the lumen of the small intestine, *T. gondii* penetrates the intestinal epithelium. Invasion of host cells by apicomplexan parasites is a parasite-driven process, and is coupled to their unique process of motility termed gliding (Sibley, 2004). Specifically, invasion is dependent on helical gliding, a corkscrew type of

movement that the parasite uses to power entry into the host cell (Håkansson *et al.*, 1999). A similar mechanism of active penetration by gliding motility may be used to cross tissue barriers to migrate into deeper tissues (Barragan and Sibley, 2002).

The ability to migrate across cellular and tissue barriers has been associated with virulence in *T. gondii*. Virulent Type I strains are able to migrate longer distances *in vitro*, and show enhanced transmigration across polarized monolayers and extracellular matrix, compared to Type II and Type III strains (Barragan and Sibley, 2002). Additionally a subpopulation of Type I parasites (true for all Type I strains tested), exhibit a long distance migration (LDM) phenotype that is not expressed in Type II or Type III strains. This trait can be upregulated, and LDM clones demonstrate enhanced transmigration *in vitro*, and faster dissemination *in vivo* (Barragan and Sibley, 2002). Analysis of transmigration across the mouse intestinal mucosa *ex vivo*, also reveals that a higher proportion of Type I parasites are able to penetrate the lamina propria and vascular endothelium compared to Type II parasites (Barragan and Sibley, 2002). Overall these data suggest that the ability to disseminate *in vivo* is linked to the enhanced motility and transmigratory capacity of virulent Type I strains. However, Type II strains, which have limited transmigratory capacity, are the most wide-spread in nature, and in a mouse model, do not take any longer than type I parasites to reach peripheral tissues (Hitziger *et al.*, 2005). This suggests that Type11 parasites may have other mechanisms for dissemination *in vivo*.

Recent work suggests that Type II and Type III strains may take advantage of host immune effector cells to enter into the circulation and disseminate to distant organs (Lambert *et al.*, 2009). This is consistent with the histological detection of Type II and

Type III parasites in leukocytes in circulation soon after infection (Courret *et al.*, 2006; Unno *et al.*, 2008). Interestingly, while adoptive transfer experiments with dendritic cells infected with parasites caused a substantial increase in the loads of Type II and Type III parasites in circulation, only a slight increase was observed with Type I parasites, suggesting that such mechanisms are less important for the dissemination of Type I parasites (Lambert *et al.*, 2009).

Thus, while Type I parasites appear to actively penetrate the deeper tissues through enhanced migratory properties, Type II and III parasites may subvert host immune cells to disseminate during infection. These differences in strategy may correlate to disease outcome and severity. Indeed Type I strains may be over-represented in transplacental infections (Fuentes *et al.*, 2001), a site of immune privilege for which parasites would have to arrive at independently.

Overview of the lytic cycle

The *T. gondii* lytic cycle can be thought of as a series of distinct steps: attachment, invasion, replication and egress. Overall these steps are very similar between the *T. gondii* tachyzoite and *Plasmodium* spp. merozoite stages, although the details may differ. Here the emphasis is on the *T. gondii* lytic cycle.

One of the defining features of apicomplexan parasites is the presence of three types of specialized secretory organelles found at the apical end of the parasite: the micronemes, rhoptries and dense granules. The sequential release of the contents of these

organelles during invasion is timed with their roles in host cell attachment, invasion and establishment of the parasitophorous vacuole, respectively (Carruthers and Sibley, 1997).

Attachment

Parasites traverse across host cell surfaces using gliding motility, which is accompanied by a low level of constitutive secretion of surface adhesins from the micronemes. Prior to invading a host cell a stronger attachment is made between the parasite and the host cell surface. This is caused by an upregulation in the cytosolic calcium levels of the parasite, and leads to the enhanced secretion of adhesins from the micronemes (Sibley, 2003). In particular, secretion of the transmembrane surface adhesin MIC2, which is indirectly connected to the parasites actin cytoskeleton via its C-terminal cytoplasmic tail, mediates attachment of the parasite to the host cell surface (Huynh and Carruthers, 2006). No specific host cell surface receptors have been identified, although interactions with host cell glycosaminoglycans and sialic acid are thought to mediate attachment (Harper *et al.*, 2004). In the initial stages of invasion, the parasite goes from a distant attachment to the host cell (typically a distance of >40 nm), to a more intimate attachment (<6 nm distance), with the apical end of the parasite becoming closely apposed to the host cell membrane (Mital *et al.*, 2005). This interaction is mediated by the microneme protein AMA1 (Mital *et al.*, 2005) and probably others, although AMA1 is necessary.

Invasion

Invasion is an active parasite driven process that is dependent on the parasite's actomyosin motor (Dobrowolski and Sibley, 1996). It is coupled to parasite helical

gliding motility, which is thought to provide the force required for the parasite to enter the host cell (Håkansson *et al.*, 1999). Following intimate attachment of the parasite to the host cell, the rhoptry organelles are secreted. Several rhoptry proteins have been shown to function in the invasion process. The complex of rhoptry neck proteins RON2/4/5/8, are inserted into the host cell plasma membrane, perhaps functioning as the specific receptors to which the parasite attaches to for rapid and efficient invasion (Besteiro *et al.*, 2009). Other rhoptry proteins anchor the moving junction that forms between the parasite and host cell as the parasite squeezes its way into the host cell (Alexander *et al.*, 2005; Lebrun *et al.*, 2005). The moving junction is thought to act as a molecular sieve, excluding specific host plasma membrane proteins from the nascent vacuole that is formed around the entering parasite, while retaining other host proteins such as GPI linked proteins (Mordue *et al.*, 1999). Completion of invasion is marked by fission of the parasitophorous vacuole from the host cell membrane at the posterior end of the parasite (Suss-Toby *et al.*, 1996). It is currently unknown what mediates this event. Invasion is a rapid process, completing within 15-20 seconds.

Replication

Since the parasite actively invades the host cell, and appears to selectively exclude specific host membrane proteins, the parasite forms its own non-fusogenic vacuole as it enters the cell. Within this protected environment, the parasite undergoes successive rounds of replication by endodyogeny, which is a specialized form of replication in which daughter cells are formed *de novo* within the mother cell (Morrissette and Sibley, 2002).

Growth and replication takes 6-8 hours (Radke *et al.*, 2001), and parasites typically undergo 6-7 rounds of replication and division before the vacuole ruptures.

Egress

Like invasion, egress out of the parasitophorous vacuole is mediated through increases in the cytosolic calcium levels of the parasite. Increases in cytosolic calcium in the parasite (which can be induced artificially using calcium ionophores) trigger microneme secretion, and release of the microneme protein perforin permeabilizes the parasite vacuole, allowing for the release of parasites from the vacuole (Kafsack *et al.*, 2009). Motile parasites subsequently leave the vacuole and host cell. Egress of parasites out of the parasitophorous vacuole may occur under a number of circumstances. During natural egress, it is thought that accumulation of the hormone abscisic acid that is produced by parasites in a density-dependent manner, leads to an increase in the cytosolic calcium levels, and initiates the cascade of events leading to egress (Nagamune *et al.*, 2008). Some studies suggest that a drop in host cell potassium concentration due to the integrity of the host plasma membrane being compromised, may be sensed by the parasite, and following changes in parasite calcium levels, may lead to parasite egress (Moudy *et al.*, 2001). Recently the ligation of death receptors on infected T-cells was found to trigger parasite egress from the infected cell (Persson *et al.*, 2007), again indicating that parasites can sense their environment and trigger their premature escape from the vacuole.

Overview of gliding motility

Gliding motility is essential for a number of different stages in the parasite life cycle. Apart from being the process by which parasites traverse host cell surfaces and cross cellular and tissue barriers, it is also critical for parasite invasion and egress, of which the former step is essential for the survival of an obligate intracellular parasite. However, in contrast to the types of motility exhibited by other motile cells, apicomplexan gliding motility is distinguished by the absence of any external appendages such as cilia, flagella, or pili, and does not involved a change in shape of the organism, as is seen during amoeboid crawling (Preston and King, 1996). Instead, apicomplexan gliding motility is a substrate-dependent type of gliding involving the translocation of an adhesin along the surface of the parasite.

When gliding is initiated, transmembrane surface adhesins such as MIC2 in *T. gondii* or the homologous TRAP protein in *Plasmodium* spp., are secreted from the micronemes at the apical end of the parasite (Carruthers *et al.*, 2000) (Figure 1). The extracellular domain of MIC2 makes contact with the host cell substrate, while the C-terminus of MIC2 extends into the parasite's cytoplasm and is connected to the parasite's actomyosin motor via the bridging protein aldolase (Jewett and Sibley, 2003). As the myosin motors push the actin filaments backwards, MIC2 is translocated rearward along the longitudinal surface of the parasite, which in turn pulls the parasite forward. At the posterior end, proteolytic cleavage of MIC2 from the surface of the parasite allows for forward migration of the parasite (Carruthers *et al.*, 2000). Gliding motility is an extremely efficient process of movement, resulting in speeds of 1-3 μm/s, at least 10 times faster than most other types of cellular motility (Sibley, 2004).

Apicomplexan parasites engage in three stereotypical gliding behaviors (Håkansson *et al.*, 1999). Helical gliding refers to the corkscrew-like motion that occurs as the parasite rotates around its longitudinal axis in a clockwise direction, and results in the forward migration of the parasite. Twirling occurs when the parasite engages in a clockwise spinning motion while being vertically oriented and attached to the substratum at the posterior end. This behavior does not result in any migration of the parasite. Circular gliding occurs when the parasite maintains the right-side of its crescent-shaped body in contact with the substratum while it undergoes counterclockwise motion. Although this behavior results in forward movement, the parasite moves in circles and so is unable to invade host cells. Only helical gliding is considered productive motility as it can result in host cell invasion (Håkansson *et al.*, 1999).

Gliding motility appears to be conserved amongst the apicomplexan parasites. In addition to *T. gondii*, gliding has been observed in *Plasmodium* and *Cryptosporidium* sporozoites, and a similar form of gliding movement is observed in the gregarines, an early branching member of the phylum (Sibley, 2004). Due to the relative ease of experimental manipulation and the availability of genetic and cellular tools, *T. gondii* is often used as a model apicomplexan for studying gliding motility (Kim and Weiss, 2004).

Subcellular localization of the glideosome

Apicomplexan parasites have a highly unique and specialized cytoskeletal architecture, which is likely a determining factor in the types of parasite movement observed. *T. gondii*

is a crescent-shaped organism, and other apicomplexan parasites such as *Plasmodium* and *Cryptosporidium* sporozoites are similarly shaped.

In *T. gondii*, the subpellicular microtubules provide the structural framework for the parasite. A group of 22 subpellicular microtubules, extend from the microtubule organizing center at the anterior of the parasite, and spiral 2/3 down the length of the parasite (Morrissette and Sibley, 2002). These function to maintain the shape and provide rigidity to the parasite framework. Closely associated with the subpellicular microtubules, and just beneath the parasite plasma membrane, is the inner membrane complex (IMC), which is composed of a closely opposed inner and outer membrane, that consists of cholesterol-rich flattened vesicles sutured together (Morrissette and Sibley, 2002). The IMC takes the same spiral shape as the subpellicular microtubules, although unlike the microtubules it extends along the entire length of the parasite. Beneath the IMC is a lattice of intermediate-like filaments that may connect the subpellicular microtubules to the IMC (Morrissette and Sibley, 2002). Embedded within the IMC are the myosin motors that function in gliding motility (Johnson *et al.*, 2007). The spiral arrangement of the IMC and subsequently the myosin motors, may play a role in the helical gliding movements undertaken by the parasite.

Glideosome components

The glideosome (Keeley and Soldati, 2004), which refers to all the molecular components required for gliding motility, is assembled in the inner membrane space that is formed

between the plasma membrane and the IMC, and provides a means to connect the actomyosin motor to the translocation of surface adhesins.

Myosin motor

Of the 11 myosin genes in *T. gondii*, the myosin A gene (TgMyoA) has been identified as the myosin motor required for gliding motility (Meissner *et al.*, 2002). It is found as a complex with the myosin light chain and the GAP45/GAP50 proteins (Gaskins *et al.*, 2004), and is firmly anchored in the inner membrane complex via the GAP45/GAP50 proteins in cholesterol-rich microdomains (Johnson *et al.*, 2007). Assembly of the motor complex may be controlled by the phosphorylation and dephosphorylation of GAP45 (Gilk *et al.*, 2009).

TgMyoA is a small, unconventional myosin belonging to the class XIV myosins (Heintzelman and Schwartzman, 1997). Biochemically it is a fast, plus-ended non-processive motor with a small tail. It has a small step size of 5.3 nm and is shown *in vitro* to translocate filaments with a velocity of 5.2 μm/s (Herm-Gotz *et al.*, 2002). TgMyoA is essential for parasite motility and survival, as shown by using a conditional knockout system (Meissner *et al.*, 2002). Suppression of TgMyoA rendered parasites non-motile, significantly impaired in invasion, and unable to escape from the parasitophorous vacuole.

Actin

The work of the motor is transduced to adhesins at the surface of the parasites via connection to actin filaments that form in the inner membrane space. Genetic studies

(Dobrowolski and Sibley, 1996) indicate that parasite actin filaments are essential for motility and invasion, although only 2-3% of parasite actin appears to be filamentous by conventional standards (sedimentation at 100,000 x g) (Dobrowolski *et al.*, 1997b; Wetzel *et al.*, 2003). Accordingly, filaments have also been difficult to detect.

T. gondii has one actin allele that has 83% identity to vertebrate actin and is expressed throughout the life cycle of the parasite (Dobrowolski *et al.*, 1997b). Parasite actin is thought to primarily function in gliding motility and related processes as actin stabilizing and destabilizing agents primarily affected these processes (Dobrowolski and Sibley, 1996; Poupel and Tardieux, 1999; Shaw and Tilney, 1999; Wetzel *et al.*, 2003). Unlike in many other organisms, actin is not thought to play a role in parasite cell division (Dobrowolski and Sibley, 1996; Shaw *et al.*, 2000). The role of actin in gliding motility is discussed in more detail in the following sections.

Aldolase

The actomyosin motor is linked to transmembrane surface adhesins via the bridging protein aldolase (Jewett and Sibley, 2003). Aldolse functions as a tetramer, and in addition to its role as a glycolytic enzyme, has been shown to have the property of crosslinking F-actin (Wang *et al.*, 1996). In apicomplexan parasites, aldolase has been shown to bind the C-terminus of the MIC2 adhesin in *T. gondii* (Jewett and Sibley, 2003), or TRAP in *Plasmodium* (Buscaglia *et al.*, 2003), that extends into the cytoplasm.

Recently, a conditional knockout line of *T. gondii* aldolase complemented with mutants defective in ATP hydrolysis or MIC2 tail binding established the essential role of aldolase in energy production in *T. gondii* (Starnes *et al.*, 2009). A 50% defect in

invasion in the absence of the aldolase-MIC2 binding suggested its essential role in efficient host cell invasion, presumably by bridging adhesin-cytoskeleton interactions in the parasite (Starnes *et al.*, 2009). No defect was observed in gliding in the mutants defective in MIC2 tail binding. It is unclear if this is because incomplete protein shutdown of aldolase may have allowed for some or all of its functions, or it may suggest the presence of additional factors involved in linkage of the cytoskeleton to surface adhesins.

MIC2

MIC2 is a transmembrane surface adhesin secreted from the micronemes at the apical end of the parasite (Carruthers *et al.*, 2000), and is the structural and functional homologue of the TRAP protein in *Plasmodium* (Kappe *et al.*, 1999). The protein has an extracellular domain containing an integrin A domain and thrombospondin repeats which bind to heparin-like molecules, and interaction with GAGs have been shown to be important for parasite binding to host cells (Ménard, 2001; Harper *et al.*, 2004). The C-terminus of MIC2 extends into the parasite cytoplasm and interacts with aldolase, providing a link from the surface adhesin to the actomyosin motor (Jewett and Sibley, 2003). MIC2 is translocated rearward across the surface of the parasite in an actin dependent manner (Carruthers *et al.*, 2000).

A conditional knockout line in MIC2 demonstrates significant defects in both attachment and invasion, and parasites are strongly impaired in helical gliding and also in twirling (Huynh and Carruthers, 2006). This implicates MIC2 as critical adhesin for productive gliding. Similarly disruption of TRAP in *Plasmodium* sporozoites results in

the inhibition of invasion and an inability to glide. Interestingly, a pendulum gliding phenotype was observed in TRAP deficient parasites (Kappe *et al.*, 1999).

Proteolytic cleavage of MIC2 from the surface of the parasite

Surface adhesins are released from the parasite during motility and invasion, and this is thought to be required for forward movement and the completion of invasion. The rhomboid intramembrane serine proteases are believed to mediate cleavage of the surface adhesins. This family of proteases is conserved in apicomplexan parasites and their activity has been demonstrated *in vitro* (Brossier *et al.*, 2005; Dowse *et al.*, 2005).

Recently, characterization of a conditional knockout in the *T. gondii* rhomboid protein ROM4, suggests that this protein functions to maintain an apical to posterior gradient of surface adhesins (Buguliskis *et al.*, 2010). Absence of ROM4 led to an increased attachment of parasites to host cells, and an increased proportion of parasites engaged in twirling motility, but parasite invasion was significantly impaired, presumably because of an inability to apically reorient.

Role of the parasite actin cytoskeleton in gliding motility and invasion

The essential role for parasite actin in gliding motility and host cell invasion was demonstrated conclusively using a genetic approach and by taking advantage of resistance to the inhibitor of actin polymerization, Cytochalasin D (CytD). By monitoring the invasion of *T. gondii* into a CytD resistant host cell line in the presence of CytD. Dobrowolski *et al.* (1996), demonstrated that host cell actin is largely dispendable for

parasite invasion. Conversely, by generating CytD resistant parasites, they showed that parasite actin filaments are essential for host cell invasion, indicating that *T. gondii* relies on its own actin cytoskeleton to power its entry into host cells. Similar experiments indicated that gliding motility also depends on parasite actin filaments, whereas host cell attachment does not (Dobrowolski and Sibley, 1996). Interestingly, the effects of CytD on parasites appear to be limited to motility dependent processes, as no effects were observed on parasite cell division or intracellular growth (Dobrowolski and Sibley, 1996; Shaw *et al.*, 2000). This suggests that parasite actin filaments primarily function during gliding motility. Subsequent studies with the actin stabilizing drug jasplakinolide (JAS), indicate that polymerization of parasite actin filaments is rate-limiting for motility, and that the turnover of parasite actin filaments is essential for productive gliding motility (Poupel and Tardieux, 1999; Shaw and Tilney, 1999; Wetzel *et al.*, 2003). Similar to CytD treatment, JAS treated parasites attach normally to host cells but cannot invade host cells (Poupel and Tardieux, 1999; Shaw and Tilney, 1999; Wetzel *et al.*, 2003). In contrast to CytD treatment, stabilizing parasite actin filaments results in increased speeds of motility, with parasites moving at rates up to 3X faster (Wetzel *et al.*, 2003). However, due to frequent reversals of direction, motility is non-productive and parasites are unable to invade host cells. These studies suggest that the control of both filament polymerization and turnover are essential for productive gliding motility and host cell invasion. The role of myosin in gliding motility and invasion was also tested in these studies, using the myosin inhibitor 2,3-butanedione monomide (Dobrowolski *et al.*, 1997a; Wetzel *et al.*, 2003). Although this inhibitor was later shown to also have non-specific effects on other aspects of cellular motility (Ostap, 2002; Yarrow *et al.*, 2003),

the essential role of myosin in gliding motility and invasion has since been firmly established using a conditional knockout system in *T. gondii* (Meissner *et al.*, 2002). Recent work indicates that the host cell cortical actin network also plays a role in efficient parasite invasion (Gonzalez *et al.*, 2009). Although the data thus far do not clearly indicate what that role is, it is possible that the host actin network functions as a scaffold to which the parasite can hook onto for efficient cell entry.

Despite the absolute requirement for parasite actin filaments during gliding motility and invasion, actin filaments are undetectable in parasites by conventional methods such as sedimentation at 100,000 x g, phalloidin staining, or conventional electron microscopy methods (Dobrowolski *et al.*, 1997b; Shaw and Tilney, 1999). However using rapid freeze-fracture electron microscopy to capture events occurring just beneath the membrane, parasite actin filaments have been visualized in the membrane footprints left behind by gliding parasites (Wetzel *et al.*, 2003; Sahoo *et al.*, 2006). This approach revealed the presence of short (0.2-1 μm in length), parallel filaments in gliding parasites. Interestingly, in JAS treated gliding parasites, filaments were of similar length but were disordered and arranged in a tangled web (Wetzel *et al.*, 2003; Sahoo *et al.*, 2006). This tangled arrangement of filaments underneath the plasma membrane in JAS treated parasites, is in contrast to the long, parallel, bundled filaments observed within the cytoplasm of JAS treated extracellular parasites, detected by conventional electron microscopy (Shaw and Tilney, 1999). While it is unclear whether these differences in the arrangement of filaments are a result of the specific techniques by which they were visualized, it is interesting to speculate that they may represent the presence of different populations of actin within the parasite, and that perhaps the shorter more numerous

filaments underneath the plasma membrane are reflective of the dynamic nature of filaments underneath the membrane during motility, that may have been captured by JAS treatment. Coincident with this, it is also intriguing to note that despite the high affinity of JAS for actin filaments (Bubb *et al.*, 1994) and its ability to inhibit parasite invasion, removal of JAS resulted in the normal invasion of parasites that had attached to host cells during JAS treatment (Shaw and Tilney, 1999), suggesting that parasites might express proteins that can rapidly turnover stabilized actin filaments. Collectively, the inability to detect parasite actin filaments in resting parasites without stabilizing agents, and the difficulty in detecting parasite filaments even in gliding parasites where they are necessary for function, suggests that parasite actin filaments are assembled transiently and rapidly turned over.

Actin binding proteins in apicomplexan parasites

In higher eukaryotes, complex actin dynamics are controlled by a multitude of actin binding proteins (Pollard and Borisy, 2003). In contrast, only a limited set of conserved actin binding proteins appears to be present in apicomplexan parasites (Baum *et al.*, 2006; Schüler and Matuschewski, 2006; Sibley *et al.*, 2007). While this does not negate the possibility that parasites might encode unique actin binding proteins (ABPs), it is interesting to note that the conserved ABPs found in apicomplexan parasites represent little more than those required to reconstitute motility *in vitro*, suggesting that this small set might be sufficient for regulating actin dynamics in the context of gliding motility. The conserved set of ABP's found in apicomplexan parasites includes formins, profilin,

Actin Depolymerizing Factor (ADF) / cofilin, capping protein, coronin and cyclase-associated protein (CAP) (Baum *et al.*, 2006; Schüler and Matuschewski, 2006). Additionally, apicomplexan parasites encode for Actin-Like Proteins (ALPs), which bear some resemblance to the diverse Actin-Related Proteins (ARPs) found in higher eukaryotes (Gordon and Sibley, 2005). *T. gondii* also uniquely expresses a novel actin binding protein named toxofilin (Poupel *et al.*, 2000; Lodoen *et al.*, 2010). Notably lacking in the parasite ABP repertoire are filament stabilizing/bundling proteins such as tropomyosin and α-actinin, strong filament severing proteins such as gelsolin, and the actin nucleation factor Arp2/3 complex (Gordon and Sibley, 2005). The absence of the Arp2/3 complex and corresponding activation factors that nucleate the formation of branched actin filaments, is consistent with the absence of such filaments in apicomplexan parasites (Wetzel *et al.*, 2003).

In vitro, the proteins demonstrated to be sufficient to reconstitute the actin based motility of the bacterium *Listeria*, are the Arp2/3 complex, profilin, ADF / cofilin, capping protein, and Arp2/3 activation factors (Loisel *et al.*, 1999). As mentioned above, the lack of Arp2/3 and corresponding activation factors in apicomplexan parasites is not surprising. Parallel actin filaments thought to form in apicomplexan parasites are likely nucleated by formin and profilin proteins in the parasite. The conserved functions of the ABPs identified above, and our current knowledge of their activity in apicomplexan parasites is described below, with special attention to the ADF/Cofilin proteins, which are the focus of this work.

Formins

Formins are large multimeric proteins characterized by the presence of an FH1 domain that has discrete polyproline regions that bind to profilin, and an FH2 domain that binds to actin (Paul and Pollard, 2009). FH2 domains promote the nucleation of free actin monomers and remain bound to the barbed end of the growing filament, while the FH1 domain recruits profilin-actin complexes, which are rapidly transferred onto the filament via processive elongation (Paul and Pollard, 2009). Nucleation of filaments by formins results in straight (rather than branched) filaments.

Plasmodium falciparum encodes for two formin-like proteins, while *T. gondii* encodes for three formin-like proteins (Baum *et al.*, 2006). All have weak FH1 domains. *P. falciparum* PfFormin1 and PfFormin2 can both nucleate heterologous actin *in vitro*, although PfFormin1 is more potent (Baum *et al.*, 2008). PfFormin1 protein expression is maximal during the late phase of the intracellular cycle of merozoite parasites, and the protein localizes to the apical end of free merozoites, partially concentrating at the moving junction during invasion (Baum *et al.*, 2008). This suggests that PfFormin1 may nucleate filaments during invasion, although it needs to be directly examined. Based on its protein expression profile, PfFormin2 is unlikely to have a role in invasion and remains to be characterized (Baum *et al.*, 2008). The localization of TgFormin1, the *T. gondii* ortholog of PfFormin1, was also found to move along the parasite periphery during invasion, but this protein has not been further characterized (Baum *et al.*, 2008).

Profilin

Profilin is a small (12-16 kDa) actin binding protein that catalyzes the exchange of nucleotide on actin monomers. When the barbed ends of filaments are capped, profilin sequesters ATP-actin monomers, keeping them in a polymerization-competent state (Pollard and Borisy, 2003). When the barbed ends of filaments are free, profilin-G-actin complexes are shuttled to the barbed ends of filaments promoting filament elongation. Profilins also act in concert with formins or the Arp2/3 complex in filament elongation.

Apicomplexan parasites express one profilin isoform. The structure of PfProfilin indicates the presence of a unique minidomain that appears to be conserved in apicomplexan profilins, although its function is unknown (Kursula *et al.*, 2008). Apicomplexan profilins appear to have similar properties to conventional profilins (Kursula *et al.*, 2008; Plattner *et al.*, 2008), although the sequestering activity is relatively weak with heterologous actin, and its interaction with homologous actin has not yet been characterized. A conditional knockout of *T. gondii* profilin established its essential role in gliding motility, invasion and egress, although the precise mechanism underlying these phenotypes has not been examined (Plattner *et al.*, 2008). TgProfilin also plays a role in the TLR11 dependent activation of the mouse innate immune system (Yarovinsky *et al.*, 2005; Plattner *et al.*, 2008).

ADF/Cofilin

The ADF / Cofilin (AC) proteins are small (15-21 kDa) actin binding proteins that are essential for stimulating actin filament turnover (Lappalainen and Drubin, 1997). Although they can interact with actin filaments in a variety of ways, the most established

activities are to cause the severing of actin filaments and/or the depolymerization of actin filaments (Bamburg, 1999). AC proteins preferentially interact with ADP-actin, and this is thought to provide a mechanism to selectively disassemble older filaments in which the nucleotide has undergone hydrolysis. Inhibiting nucleotide exchange on G-actin is a defining characteristic of AC proteins.

All apicomplexan parasites have one AC isoform, except *Plasmodium,* which has two (Schuler *et al.*, 2005). Preliminary characterization of *T. gondii* ADF reveals that it can cause the net disassembly of heterologous actin filaments, and that it binds to G-actin (Allen *et al.*, 1997). The AC family of proteins will be discussed in more detail in the next section.

Capping Protein

Capping protein is a heterodimeric protein consisting of an α and β subunit, each of approximately 30 kDa. The dimeric complex tightly binds to the barbed ends of filaments and stops the growth of filaments (Cooper and Schafer, 2000). In this way it is thought to funnel actin monomers to new growing filaments that are uncapped.

Apicomplexan parasites appear to have one copy each of both subunits (Schüler and Matuschewski, 2006). Recombinant *Plasmodium berghei* heterodimer capping protein is able to cap heterologous filaments *in vitro* (Ganter *et al.*, 2009). A genetic knockout in the β subunit in *P. berghei* results in motility defects in sporozoites, resulting in their inability to colonize the salivary glands in the insect vector and therefore an inability to complete the life cycle (Ganter *et al.*, 2009).

Coronin

Coronin is an approximately 55 kDa WD40 repeat protein that consists of a β-propeller domain, a unique region that is highly variable in sequence and length, and a coiled-coil domain (Gandhi and Goode, 2008). It is known to bind to F-actin but its mechanism of activity has been unclear. Recent work suggests that coronin protects new ATP-rich filaments from disassembly by ADF/cofilin proteins, and recruits Arp2/3 complex to these sites for filament growth (Gandhi and Goode, 2008). In contrast, at the rear end of lamellipodia coronin synergizes with ADF/cofilin proteins to disassemble old ADP-rich filaments (Gandhi and Goode, 2008).

Most apicomplexan parasites have one coronin homologue (Baum *et al.*, 2006; Schüler and Matuschewski, 2006). In *Plasmodium falciparum*, a protein in parasite extracts that reacts with a coronin antibody has been shown to associate with F-actin (Tardieux *et al.*, 1998). Expression of a fluorescently tagged coronin has also been reported to be apically localized in *Plasmodium* merozoites (cited as unpublished data in (Baum *et al.*, 2006)). Overall, very little is known about the function and activity of coronin in parasites.

Cyclase Associated Protein (CAP)

Cyclase-Associated Protein (CAP) is a large multi-domain protein that includes an N-terminal helical folded domain that has been shown to bind to ADF/cofilin-bound ADP monomers; a C-terminal beta sheet domain that binds ADP-actin monomers; and a central domain with proline rich regions and a WH2 domain that is necessary for catalyzing the nucleotide exchange of ADP-actin to ATP actin in the presence of

ADF/cofilin (Chaudhry *et al.*, 2010). Thus, CAP appears to function to recharge actin monomers in the presence of ADF/cofilin, thus contributing to enhanced filament turnover.

Apicomplexan parasites have one CAP homologue, but in contrast to other characterized CAPs, they only contain the C-terminal actin binding domain (Hliscs *et al.*, 2010). *Cryptosporidium parvum* CAP was recently found to sequester heterologous actin monomers *in vitro*, indicating that it likely functions as a monomer sequestering protein. (Hliscs *et al.*, 2010). *In vivo*, a genetic knockout of CAP in *Plasmodium berghei* prevented the maturation of oocysts in the insect vector, and was functionally complemented by the expression of *C. parvum* CAP (Hliscs *et al.*, 2010). These data suggest an essential role for CAP in sequestering actin monomers in *Plasmodium* oocyst development.

Actin-Like Proteins (ALPs)

Apicomplexan parasites appear to lack the Arp2/3 complex found in higher eukaryotes which nucleates the polymerization of branched filament networks (Gordon and Sibley, 2005). However, other Arp homologues are conserved in apicomplexan parasites, and these are presumed to function in vesicular transport and chromatin remodeling, although they are as yet uncharacterized (Gordon and Sibley, 2005). In addition to these proteins, apicomplexan parasites encode for an additional seven related proteins that have been named actin-like proteins (ALPs) (Gordon and Sibley, 2005). ALP1 was recently characterized and found to play a role during daughter cell division in *T. gondii* (Gordon *et al.*, 2008; Gordon *et al.*, 2010).

Toxofilin

Toxofilin is a 27 kDa protein that appears to be unique to *T. gondii* (Poupel *et al.*, 2000). It is a rhoptry protein that is secreted into host cells during invasion (Lodoen *et al.*, 2010). *In vitro*, it prevents the polymerization of actin, and inhibits the disassembly of actin filaments, suggesting that it can sequester actin monomers and cap filaments respectively (Poupel *et al.*, 2000). Expression of toxofilin in mammalian non-muscle cells disrupts cytoplasmic stress fibers (Poupel *et al.*, 2000). Co-crystallization of toxofilin with heterologous actin reveals that it stabilizes an anti-parallel formation of actin dimers and inhibits their nucleotide exchange (Lee *et al.*, 2007). Surprisingly, a recent genetic knockout in toxofilin demonstrated no defects in the parasite lytic cycle *in vitro*, including host cell invasion and intracellular growth (Lodoen *et al.*, 2010).

This limited set of proteins constitutes the actin binding proteins that have been identified in apicomplexan parasites to date. Interestingly, while bundling and strong severing proteins are absent, there is a bias towards G-actin binding proteins, perhaps accounting for the lack of filamentous actin in parasites under normal conditions. However, during gliding motility, filaments are predicted to be transiently assembled and rapidly turned over. To examine the role of filament turnover in regulating actin dynamics in apicomplexan parasites, we chose to focus on the role of the ADF / Cofilin family of proteins.

ADF/Cofilin proteins

The ADF/Cofilins are a well-studied family of proteins that are found ubiquitously in eukaryotes. They are essential in all systems examined to date and have a conserved structure and function (Bamburg, 1999). AC proteins function to accelerate actin filament turnover (Lappalainen and Drubin, 1997), and are thought to be singly responsible for the high rates of filament turnover observed *in vivo* (Carlier *et al.*, 1997). Their ability to drive filament turnover stems from directly causing filament disassembly without capping filament ends (Ono, 2007). This increases the pool of actin monomers for polymerization, and depending on the mechanism of filament disassembly, increases the number of filaments with free barbed ends, allowing for the rapid polymerization of new filaments. The preferential interaction of AC proteins with ADP-actin is thought to provide a clock mechanism to selectively disassemble older filaments, releasing actin monomers for the polymerization of new filaments.

AC proteins concentrate at the leading edge of motile cells, and in other regions of the cell undergoing dynamic actin turnover (Bamburg and Bray, 1987; Yonezawa *et al.*, 1987; Aizawa *et al.*, 1995). Studies in which the activity of AC proteins has been downregulated or selectively inactivated in motile cells, indicate it plays a critical role in persistent migration and in determining the direction of cellular movement (Ghosh *et al.*, 2004; Hotulainen *et al.*, 2005). Overexpression of AC proteins enhances cell migration or motility in a variety of systems (Aizawa *et al.*, 1996; Meberg and Bamburg, 2000; Yap *et al.*, 2005).

Single-celled organisms express either one ADF or cofilin gene, while higher eukaryotes have multiple isoforms, that are often expressed in a cell-type/tissue-specific manner. Genetic knockouts are lethal where only one isoform exists, and where multiple isoforms are expressed, at least one is essential for viability (Ono, 2007). This highlights the slightly different functions varying isoforms may have, depending on the cellular context in which they are expressed. The absence of AC proteins also leads to cytokinesis defects in a number of systems (Abe *et al.*, 1996; Ono *et al.*, 2003; Hotulainen *et al.*, 2005).

Structure of AC proteins

The structures of a number of AC proteins from a variety of kingdoms have been solved, and appear to be well conserved (Hatanaka *et al.*, 1996; Fedorov *et al.*, 1997; Leonard *et al.*, 1997; Bowman *et al.*, 2000; Pope *et al.*, 2004). They consist of an internal core of 4 or 5 β-sheets, surrounded by 4 or 5 α-helices. Nonhomologous sequences between the most divergent members of the family occur in loops between the conserved secondary structure elements (Bamburg, 1999). Sites required for actin binding have been mapped by mutagenesis (Moriyama *et al.*, 1992; Lappalainen *et al.*, 1997; Moriyama and Yahara, 1999; Ono *et al.*, 1999), cross-linking (Yonezawa *et al.*, 1991), peptide competition or synchotron electron footprinting (Guan *et al.*, 2002). Actin binding sites can be classified as those required for binding to both monomeric and filamentous actin, and those required exclusively for F-actin binding (Lappalainen *et al.*, 1997). Sites required for binding to both G- and F-actin are located in the longest α-helix (α-helix 3 or 4

depending on the isoform), and at the N-terminus. Specific basic residues in the long α-helix are highly conserved and have been identified as directly interacting with the filament. The N-terminus also contains the conserved serine residue, which has been identified as a physiologically important phosphoregulatory site in many systems (Ono, 2007). Sites required exclusively for binding to the filament have been identified in the "F-loop", which consists of strands β4 and β5 and projects out of the structure (Lappalainen *et al.*, 1997; Pope *et al.*, 2000), and charged residues in the C-terminal α-helix (Lappalainen *et al.*, 1997) or the C-terminal tail (Ono *et al.*, 1999).

Based on electron microscopy studies, AC proteins are thought to bind to the filament between actin subdomains 1 and 3, which results in the change in the helical structure of the filament, either by a change in twist of the filament (McGough *et al.*, 1997), or by stabilizing a tilted conformation of the filament (Galkin *et al.*, 2001; Galkin *et al.*, 2003). Changes in the local filament structure including weakening of both longitudinal and lateral contacts are thought to destabilize the filament and may result in fragmentation (McGough *et al.*, 1997; Paavilainen *et al.*, 2008; Pfaendtner *et al.*, 2010).

AC activities in vitro / Mechanisms of filament turnover activity

AC proteins exhibit a wide variety of activities *in vitro*. These include binding to G-actin in a 1:1 stoichiometry, inhibiting nucleotide exchange on G-actin, causing the disassembly of actin filaments, and nucleating new filaments (Bamburg, 1999; Andrianantoandro and Pollard, 2006). By far the most important activity in terms of their function in increasing actin turnover, is their filament disassembly activity.

The most established mechanisms for AC-mediated filament disassembly are the severing of filaments, and the enhanced pointed-end depolymerization of actin filaments. Severing involves the binding of the AC protein along the side of the filament (McGough *et al.*, 1997). This causes a change in the helical twist of the filament (McGough *et al.*, 1997) or stabilizes a tilted conformation of the filament (Galkin *et al.*, 2001; Galkin *et al.*, 2003), resulting in increased local lateral, longitudinal and torsional strain on the filament, which may lead to fragmentation of the filament (Pfaendtner *et al.*, 2010). Enhanced pointed-end depolymerization is thought to occur by binding of the AC protein to the terminal subunits of the filament, which could increase the torsional strain at this site causing the release of the terminal subunit (Carlier *et al.*, 1997). As mentioned earlier, since AC proteins interact with filaments without capping them, either mechanism will increase the pool of actin monomers. Severing is believed to be the predominant mechanism although the two are not mutually exclusive. Another property shared by a small subset of AC proteins is the ability to sequester actin monomers and prevent their polymerization (Yamashiro *et al.*, 2005).

Regulation of AC activity

In a number of systems, AC proteins are negatively regulated by phosphorylation on a conserved N-terminal serine residue, most commonly serine 3 in animals (Agnew *et al.*, 1995; Moriyama *et al.*, 1996; Meberg *et al.*, 1998), and serine 6 in plants (Smertenko *et al.*, 1998). Specific kinases, LIMK and TESK in mammals (Arber *et al.*, 1998; Yang *et al.*, 1998; Toshima *et al.*, 2001), and CDPK in plants (Allwood *et al.*, 2001), and specific

phosphatases, slingshot (Niwa *et al.*, 2002) and chronophin (Gohla *et al.*, 2005), mediate the phosphorylation and dephosphorylation reactions. Interestingly, phosphorylation does not result in any structural change of the protein (Blanchoin *et al.*, 2000). Instead, since the N-terminus is an important actin binding site interacting with both F- and G- actin, phosphorylation at this site reduces the affinity of the interaction. However, phosphorylation has been shown not to be important for controlling cofilin activity in yeast, based on the absence of a phenotype in cells expressing a non-phosphotylatable mutant cofilin (Lappalainen *et al.*, 1997).

AC interactions with F- and G- actin can be regulated by other actin binding proteins. Tropomyosin is a highly conserved actin binding protein that binds to the sides of actin filaments and protects them from AC-mediated disassembly (Ono, 2007). Similarly coronin was recently reported to bind and protect ATP-rich filaments from AC-mediated destruction (Gandhi and Goode, 2008). On the other hand, CAP and profilin may compete with AC proteins for interacting with G-actin (Ono, 2007). Phosphoinositides have been shown to bind to a variety of actin binding sites on AC proteins and compete with actin for binding (Ojala *et al.*, 2001; Gorbatyuk *et al.*, 2006). While this is a potential regulatory mechanism, it has not been validated *in vivo*. pH is another mechanism proposed to regulate AC activity *in vivo*. Most, but not all, AC proteins show pH sensitive activity *in vitro*, causing the net depolymerization of filaments at more alkaline pH's (Hawkins *et al.*, 1993; Hayden *et al.*, 1993; Chen *et al.*, 2004). There is some evidence to suggest that local pH changes within the cell may regulate AC activity (Bernstein *et al.*, 2000).

AC proteins in apicomplexan parasites

AC proteins are conserved in apicomplexan parasites where they have been named ADF. Most parasites express one isoform, except for *Plasmodium*, which expresses two (Baum *et al.*, 2006; Schüler and Matuschewski, 2006). A preliminary report on *T. gondii* ADF (TgADF) provided some basic characterization of the protein, indicating that it could cause the net disassembly of heterologous actin filaments, and bind to G-actin (Allen *et al.*, 1997). It also showed that TgADF partially localized underneath the plasma membrane, sometimes colocalizing with actin, suggesting that TgADF may have a role in regulating actin dynamics at this site. These data suggested that TgADF had the conserved function and may play a role in regulating filament turnover.

More recently the characterization of *Plasmodium* ADF1, which is essential in bloodstage parasites, revealed that it had some unique properties. PfADF1 was unable to interact with F-actin, having no effect on filament disassembly or assembly, and it had a weak affinity for ATP-G-actin (Schuler *et al.*, 2005). Unlike all ADFs characterized up until that time, it did not inhibit nucleotide exchange on G-actin, and instead caused a modest increase in exchange activity, a property more typically attributed to profilin. However, recently *Tetrahymena* ADF was also shown to increase nucleotide exchange on G-actin (Shiozaki *et al.*, 2009). The second *Plasmodium* ADF isoform, PfADF2, was recently shown to have a role in the insect vector with the genetic knockout having developmental defects (Doi *et al.*, 2010). ADF has also been shown to be expressed in the apicomplexan parasite *Eimeria tenella*, and was shown to bind to parasite actin (Xu *et al.*, 2008). While the data on *Plasmodium* ADFs indicate the *in vivo* importance of ADF in

apicomplexan parasites, the unusual biochemical properties of PfADF1 calls into question what function the protein has in the parasite, and whether any of these properties are more broadly typical of apicomplexan ADFs, or if they are restricted to PfADF1. More detailed analyses of TgADF biochemical activities will be required to determine the spectrum of its interactions with actin, and to determine if they play a role in regulating gliding motility.

Parasite actins

In recent years our understanding of the nature of parasite actins has advanced, particularly with the ability to purify recombinant parasite actin. We now understand that the properties of apicomplexan parasite actins are intrinsically different compared to actins from higher eukaryotes.

Plasmodium actin isolated from parasites (Schmitz *et al.*, 2005), or recombinantly expressed TgACT (Sahoo *et al.*, 2006), polymerizes into filaments of an average length of 0.1 μm *in vitro*. This is in sharp contrast to rabbit actin, which under the same conditions polymerizes filaments of 2-4 μm in length (Sahoo *et al.*, 2006). These data indicate that parasite actin is intrinsically unstable, and this property appears to be conserved amongst the apicomplexan parasites. Interestingly, even when filaments are stabilized with phalloidin or jasplakinolide, parasite actin filaments do not grow longer than 0.5 μm, suggesting that there are features in their molecular architecture that limit their length (Sahoo *et al.*, 2006). Many of the divergent residues in parasite actins are found at the actin monomer face (Sahoo *et al.*, 2006). Consistent with this is the finding

that *T. gondii* actin is unable to co-polymerize with vertebrate actin despite having an apparent critical concentration 3-4 fold lower than conventional actins (Sahoo *et al.*, 2006).

Together these data indicate the unique nature of the apicomplexan actin cytoskeleton, and again suggest that the system is optimized for the rapid assembly and disassembly of actin filaments, and must be tightly regulated.

Aim and scope of thesis

Apicomplexans utilize a unique process of motility termed gliding to traverse host cell surfaces and navigate through tissue and cellular barriers. Gliding motility is intimately coupled to the processes of host cell invasion and host cell egress, all of which critically depend on the parasite's actin cytoskeleton. While the molecular details of the other components of the glideosome are beginning to be filled in, relatively little is known about the parasite actin itself, or the regulation of its dynamics.

Apicomplexan parasites express a limited set of actin binding proteins, for which much is known about their activities from other systems, and whose functions appear to be conserved even when examined across kingdoms. Despite the discovery that parasite actins are short and transient, none of the conserved actin binding proteins stand out as filament stabilizing proteins. Instead this small set of actin binding proteins appears to be directed towards ensuring the rapid turnover of actin, disassembling/limiting the growth of old filaments, and funneling monomers towards the polymerization of new filaments. This is consistent with the view that rapid cycles of filament assembly and disassembly

are required during gliding motility. In all other experimental systems, the ADF/Cofilin proteins are established as being essential for the turnover of actin filaments, and are thought to be singly responsible for the high rates of actin turnover that are observed within dynamic regions of the cell. The goal of this thesis was to define the role of the ADF/Cofilin proteins in *T. gondii.*

We hypothesized that ADF would have an essential role in apicomplexan gliding motility (Figure 1), since parasite actin filaments have a demonstrated function in this process. To determine how ADF might regulate parasite actin dynamics, we first defined its interactions with actin *in vitro*. Previous work had indicated that TgADF caused the net disassembly of actin filaments and could bind to G-actin. However, those experiments were done with heterologous actin. Given that we now know parasite actin is intrinsically different, it was important to examine the interactions of TgADF with its native substrate. Therefore in our experiments we examined the interactions of TgADF with conventional eukaryotic actin as well as homologous *T. gondii* actin.

While an earlier report had indicated that TgADF caused the net disassembly of actin filaments, there was no examination into the mechanism by which this occurred. Furthermore, AC proteins can influence the activities of actin in a number of ways, yet none of these interactions had been explored. We conducted a detailed examination of the interactions of TgADF with heterologous and homologous G- actin and F-actin to precisely define the activities of TgADF. We then examined the impact of the absence of TgADF on the parasite life cycle, by generating a parasite line in which the expression of TgADF could be suppressed. We found that TgADF plays an essential role in a number of steps in the parasite life cycle. What follows in the chapters ahead, are the findings that

lead us to surmise the mechanisms by which ADF interacts with parasite actin, and its role in regulating gliding motility.

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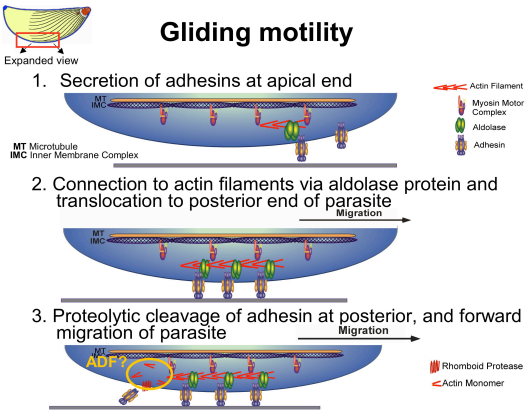
A.



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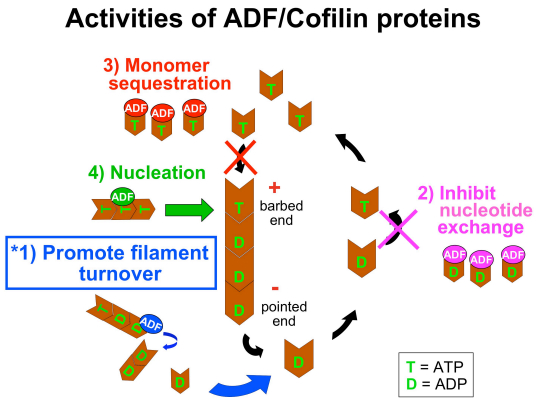

Figure1. Models of gliding motility and activities of ADF/Cofilin proteins. A) Model of gliding motility in apicomplexan parasites. The cartoon depicts the events occurring in the parasite inner membrane space during gliding motility. A putative role for ADF in filament turnover is depicted in step 3. B) Cartoon depicting the different ways in which ADF/Cofilin proteins can regulate actin dynamics. Filament turnover is thought to be their most important activity.

Chapter 2

Preliminary characterization of *Toxoplasma gondii* Actin Depolymerizing Factor

PREFACE

Work presented in this chapter was conducted by Simren Mehta and Keliang Tang. I provided the TgADF protein and Keliang purified the TgACT protein and performed the TgACT experiments presented in Figure 4.

This chapter was composed entirely by Simren Mehta. Comments from L. David Sibley were incorporated into the final version presented here.

This chapter formed the foundation for subsequent chapters and is not intended for publication.

ABSTRACT

Apicomplexan parasites utilize a unique process of motility that is dependent on parasite actin filaments. Interestingly, 98% of parasite actin is unpolymerized suggesting that filaments are rapidly assembled and turned over. The Actin Depolymerizing Factor (ADF)/Cofilin family of proteins are ubiquitously found in eukaryotes and are essential for promoting actin filament turnover. ADF is one of the few actin binding proteins conserved in apicomplexan parasites. Previous work demonstrated that *Toxoplasma gondii* ADF (TgADF) causes the net disassembly of rabbit actin filaments, and complexes with rabbit actin monomers *in vitro*. In this study we extend these initial findings to further characterize TgADF.

By immunofluorescence microscopy, ADF and actin relocalized from the parasite cytosol to underneath the plasma membrane during motility, consistent with the relocalization of ADF/Cofilin proteins to regions of high actin turnover. Interestingly we found the ratio of ADF : actin in the parasite to be almost equimolar. Direct observation of TgADF activity by fluorescence microscopy demonstrated that TgADF disassembled recombinant *T. gondii* actin filaments in a time-dependent manner. Mutation of the conserved serine 3 residue in TgADF to glutamic acid indicated the N-terminus of TgADF is important for interacting with *T. gondii* actin. Isolation of TgADF from parasites revealed that TgADF is post-translationally modified *in vivo*, although the role of such modifications remains uncertain.

The preliminary characterization of TgADF presented here and the tools that were generated, formed the foundation for the more detailed assessment of TgADF activity and function that follows in the subsequent chapters.

INTRODUCTION

Gliding motility is a unique process of motility that is conserved in apicomplexan parasites. It involves the connection of the parasites actomyosin cytoskeleton with the translocation of parasite surface adhesins that make contact with host cell surfaces. Movement of the myosin motor along parasite actin filaments is thought to result in the rearward translocation of the adhesin MIC2 along the surface of the parasite. Cleavage of the adhesin at the posterior end of the parasite allows for forward migration of the parasite (Sibley, 2004).

A critical role for the parasite's actin cytoskeleton in driving gliding motility and the coupled process of host cell invasion, has been shown genetically (Dobrowolski and Sibley, 1996) and through the use of inhibitor studies (Dobrowolski and Sibley, 1996; Poupel and Tardieux, 1999; Shaw and Tilney, 1999; Wetzel *et al.*, 2003). For example, parasites treated with the actin destabilizing agent cytochalasin D are rendered non-motile, and although they can attach to host cells, they are unable to invade (Dobrowolski and Sibley, 1996), indicating that parasite actin polymerization is essential for gliding motility and invasion of host cells. In contrast, treatment of parasites with low levels of the actin stabilizing agent jasplakinolide, results in parasites that move faster (upto three times faster), but since they undergo frequent reversals in direction, parasites are unable to pursue forward directional movement and unable to invade host cells (Wetzel *et al.*, 2003). These findings indicate that polymerization of parasite actin is rate-limiting for motility, and suggest that parasite actin filament turnover is essential for productive motility.

Despite the requirement for parasite actin filaments during gliding motility and invasion, 98% of the actin in apicomplexan parasites is unpolymerized (Dobrowolski *et al.*, 1997), and parasite actin filaments are difficult to detect using conventional methods (Shaw and Tilney, 1999). Using rapid freeze-fracture electron microscopy, actin filaments have been detected in membrane footprints of gliding parasites (Wetzel *et al.*, 2003; Sahoo *et al.*, 2006), suggesting that parasite actin filaments are only formed transiently under the plasma membrane during gliding motility. However, in contrast to higher eukaryotes, which express a multitude of actin binding proteins to regulate complex actin dynamics, only a small set of these actin binding proteins are found in the genomes of apicomplexan parasites (Baum *et al.*, 2006; Schüler and Matuschewski, 2006), and the presence of functional homologues has not yet been investigated. The conserved set of actin binding proteins consists of formins, profilin, actin depolymerizing factor (ADF), capping protein, coronin and cyclase-associated protein, and represents little more than the minimal set of actin binding proteins required to reconstitute actin-based motility *in vitro* (Loisel *et al.*, 1999). Interestingly, this repertoire is biased towards actin monomer binding proteins. Other actin binding proteins involved in severing, cross-linking and bundling actin filaments are absent in apicomplexan parasites (Baum *et al.*, 2006; Schüler and Matuschewski, 2006). Based on studies from higher eukaryotic systems, only ADF has the essential function of increasing actin filament turnover.

The ADF/Cofilin (AC) family of proteins are found ubiquitously in eukaryotes, and have a highly conserved structure and function (Bamburg, 1999). Proteins of this family are essential for increasing actin filament turnover (Lappalainen and Drubin, 1997), and are observed to localize to the leading edge of motile cells and other areas of

high actin turnover within the cell (Bamburg and Bray, 1987; Yonezawa *et al.*, 1987; Aizawa *et al.*, 1995). AC proteins bind to both monomeric and filamentous actin, and display a variety of activities including increasing the depolymerization rate of actin filaments (Carlier *et al.*, 1997), severing filaments (Blanchoin and Pollard, 1999), nucleating new filaments at high concentrations (Andrianantoandro and Pollard, 2006), and sequestering actin monomers (Yamashiro *et al.*, 2005). Severing actin filaments is thought to be the main mechanism by which AC proteins cause the rapid turnover of actin filaments (Andrianantoandro and Pollard, 2006), and is presumed to result from the local change in the helical twist of the filament that occurs when AC proteins bind to F-actin (McGough *et al.*, 1997).

AC proteins are conserved in apicomplexan parasites, in which they are named ADF, and most parasites express a single isoform, except for *Plasmodium*, which expresses two isoforms. Previous work on *Toxoplasma gondii* ADF (TgADF) (Allen *et al.*, 1997) demonstrated that it caused the net disassembly of rabbit actin filaments in actin sedimentation assays, suggesting that TgADF has maintained the property of filament disassembly. When localized in parasites, TgADF was observed to be cytoplasmic and to stain the rim of parasites, and by immunoelectron microscopy was found in clusters underneath the parasite membrane, which occasionally co-localized with actin. Finally, using gel filtration they demonstrated that TgADF could form a complex with rabbit actin monomers. While this preliminary report established that TgADF had some of the properties of AC family members, there was no investigation into the mechanism by which TgADF caused the net disassembly of actin filaments, or any detailed examination of how it interacted with actin filaments, particularly *T. gondii*

actin filaments which appear to be less stable than rabbit or other higher eukaryotic actins. Possible mechanisms regulating TgADF activity were also not addressed.

Regulation of AC protein activity is primarily thought to be controlled by phosphorylation on a conserved N-terminal serine residue, most commonly serine 3 in animals (Agnew *et al.*, 1995; Moriyama *et al.*, 1996; Meberg *et al.*, 1998) and serine 6 in plants (Smertenko *et al.*, 1998), which inhibits the interaction of the AC protein with actin. In higher eukaryotes phosphorylation is regulated by the LIM kinases (Arber *et al.*, 1998; Yang *et al.*, 1998) or TES kinases (Toshima *et al.*, 2001), while in plants it is mediated by the CDPK family of kinases (Allwood *et al.*, 2001). While apicomplexan parasites do not possess a homologue for LIMK, they express many homologues to the plant-like CDPK's, some of which are known to regulate parasite gliding motility and invasion (Kieschnick *et al.*, 2001; Billker *et al.*, 2009). As the N-terminus of AC proteins is important for binding to both G- and F- actin (Lappalainen *et al.*, 1997), this site is highly conserved amongst AC proteins, even in family members whose activity does not appear to be regulated by phosphorylation (Lappalainen *et al.*, 1997; Bamburg, 1999). Although the serine 3 residue is conserved in apicomplexan parasites, it is unknown whether apicomplexan ADF activity is regulated by phosphorylation.

In this study we extended the initial observations of Allen *et al*. (1997), on the activity of TgADF, and generated the tools and reagents that will be required to more fully address the activities and functions of TgADF in the parasite. We examined the localization of TgADF during motility using immunofluorescence, and evaluated the interaction of TgADF with *T. gondii* actin (TgACT). Lastly we examined the possibility of phosphorylation as a means of regulating TgADF activity in the parasite. Overall the

findings in this chapter provided a foundation for the more detailed analyses of TgADF activity and function that are examined in the later chapters of this thesis.

MATERIALS AND METHODS

Cloning, expression and antibody production to TgADF

TgADF was amplified from RH strain *T. gondii* cDNA using primers ADF FP1 and ADF RP1 (see supplemental Table S1) and cloned into the pET22b+ vector (Novagen, Darmstadt, Germany) at the Nde1-Xho1 site, which resulted in the addition of a C-terminal His_{6X} tag. Recombinant TgADF protein was expressed in *E. coli* BL21 cells following overnight induction with 1 mM IPTG at 25^{o}C, and the soluble fraction was purified using Ni-NTA agarose according to manufacturer's recommendation (Invitrogen, Carlsbad, CA). The purified protein was stored in G-buffer (5 mM Tris-HCl pH 8.0, 0.2 mM $CaCl_2$, 0.2 mM ATP, 0.5 mM DTT). A rabbit polyclonal antibody to recombinant TgADF was commercially generated (Covance Research Products, Inc., Denver, PA) and this antibody was subsequently used in almost all assays to detect ADF, with the exception of the immunofluorescence assay below in which a previously generated rat anti-TgADF antibody was used.

Localization of ADF and actin in T. gondii by immunofluorescence microscopy

To prepare intracellular parasites, HFF cells seeded on coverslips were infected with freshly egressed tachyzoites for 24 hr at 37^{o}C. Extracellular non-motile parasites were prepared by centrifuging freshly harvested tachyzoites onto BSA-coated coverslips. Parasites were kept non-motile by performing procedures at 18^{o}C, so that surface adhesins necessary for gliding were not secreted. Gliding parasites were prepared the same way as extracellular parasites except that coverslips were incubated at 37^{o}C for 15

min to allow parasites to glide before fixing cells. In all cases, coverslips were fixed with 4% paraformaldehyde and permeabilized with 0.25% Triton X-100. Parasites were stained with rat anti-TgADF and rabbit anti-TgACT primary antibodies followed by secondary antibodies conjugated to Alexa Fluor 488 or Alexa Fluor 594 (Molecular Probes, OR), respectively. Images were acquired using a Zeiss Axioskop 2 MOT Plus microscope equipped with a 63X 1.3 numerical aperture lens and an AxioCam Mrm camera (Carl Zeiss, Inc.; Thornwood, NY). Z-stacks were collected by wide-field immunofluorescence microscopy and images were deconvolved with Axiovison software using the nearest neighbor algorithm. LSM510 software was used to merge 4-5 center slices and generate profiles of staining intensity at points indicated on parasites.

Quantitation of ADF concentration in T. gondii

Freshly egressed parasites were harvested and extracted for 30 min at room temperature in actin stabilization buffer (60 mM PIPES, 25 mM HEPES, 10mM EGTA, 2mM $MgCl_2$, 125 mM KCl) containing 0.5% Triton X-100 and protease inhibitor cocktail (E64, 1 μg/ml; AEBSF, 10 μg/ml; TLCK, 10 μg/ml; leupeptin, 1 μg/ml). Following boiling, samples were diluted in protein gel loading buffer and resolved by 15% SDS-PAGE. To calculate the amount of ADF that was in parasite lysate, known amounts of recombinant TgADF protein were also loaded on the gel and used to generate a standard curve. ADF was detected in parasite lysate by western blotting with a rabbit anti-TgADF antibody followed by a peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). Chemiluminescence with ECL Plus (GE Healthcare,

Piscataway, NJ) was used for detection, and bands were quantified using an FLA-5000 phosphorimager (Fuji Film Medical Systems; Stamford, CT).

To estimate the concentration of ADF in *T. gondii*, the shape of a tachyzoite was described as an oblate spheroid with dimensions of 7 μm length (2a) and 2 μm width (2b) and a volume of 4/3 $\pi a \times b^2$ or 1.47×10^{-11} ml.

Thus molarity was calculated as:

$$\text{Molarity (M)} = \frac{\text{protein concentration per cell in grams}}{\text{molecular weight in grams} \times \#\text{ of cells} \times 1.47 \times 10^{-14}\text{ liter}}$$

Actin filamentation assay

T. gondii actin and wild-type or mutant ADF containing the serine to glutamic acid mutation at residue 3, were purified as previously described (Sahoo *et al.*, 2006; Mehta and Sibley, 2010). Actin (5μM) was polymerized by the addition of $1/10^{th}$ volume of 10X F-buffer (500 mM KCl, 20 mM $MgCl_2$, 10 mM ATP) in the presence of 5 μM unlabeled phalloidin (Molecular Probes, Eugene, OR), 0.33 μM Alexa Fluor 488-labeled phalloidin (Molecular Probes, Eugene, OR) and 50 μM TgADF. After 1 hr the samples were placed on a slide and examined by fluorescence microscopy using a Zeiss Axioplan microscope equipped with a 63X 1.3 numerical aperture lens. Images were collected with an AxioCam cooled CCD camera directed by Axiovision software (Version 4.5).

Generation of parasite clones expressing serine 3 mutant ADF alleles

TgADF (GenBank accession number AAC47717) was PCR amplified to include a C-terminal HA9 tag (primer sequences given in supplemental Table S1) and cloned into the

T. gondii cloning vector pBlueScript-SAG/CAT/SAG using the HindIII and PacI restriction sites. This plasmid was further digested with Ecor1 to insert the genomic sequence 1 kb upstream of the TgADF start site amplified from RH strain genomic DNA (Table S1), to drive expression of the *ADFHA9* gene, resulting in the plasmid ADF/ADFHA9/SAG1. Point mutations were introduced into *ADFHA9* using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA) and specific primers (Table S1), and the sequence was verified by DNA sequencing.

The wild-type, S3A or S3E ADF/ADFHA9/SAG1 plasmids were co-transfected with a plasmid encoding the *ble* selectable marker (Messina *et al.*, 1995) into RH strain parasites. After selection for stable transformants parasites were single-cell cloned by limiting dilution. Clones were screened by western blotting for TgADF as described above, and clones that had the highest relative expression of ADFHA9 relative to endogenous ADF, were pursued in subsequent assays.

Invasion assay

Invasion of HFF monolayers seeded on coverslips was detected using the two-color staining protocol to distinguish extracellular from intracellular parasites and done essentially as previously described (Buguliskis *et al.*, 2010) with the following minor modifications. Parasites were resuspended in Dulbecco's Modified Eagles Medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 2 mM glutamine, 20 mM HEPES (pH 7.5) and allowed to invade host cells for 10 min at 37^{o}C. To detect intracellular parasites, cells were permeabilized with 0.25% Trition X-100 before staining.

Plaque assay

Plaque assays were performed as previously described (Roos *et al.*, 1994). Confluent HFF monolayers seeded in 6-well plates were infected with 300 parasites and left undisturbed for 8 days at 37^{o}C. Monolayers were fixed 8 days after infection and stained with 0.1% crystal violet.

Gliding assay

Gliding assays were performed as previously described (Starnes *et al.*, 2009). Briefly, freshly egressed parasites were allowed to glide on BSA-coated coverslips for 15 min at 37^{o}C. Coverslips were fixed with 4% paraformaldehyde and permeabilized with 0.25% Triton X-100, followed by staining with rabbit anti-TgADF and mouse anti-SAG1 antibodies, as described above.

Immunoprecipitation

Recombinant protein G sepharose beads (Pierce, Rockford, IL) were coated with rabbit anti-TgADF antibody for at least an hour at 4^{o}C. Parasites (5 x 10^{7} –1 x 10^{8} per immunoprecipitation) were harvested and resuspended in RIPA buffer (50 mM Tris pH7.4, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 5mM EDTA) supplemented with phosphatase inhibitors 50 mM sodium fluoride and 10 mM sodium orthovanadate, 5 mM EDTA, protease inhibitor cocktail, and Dnase1. After extraction for at least 1 hour at 4^{o}C, the insoluble material was removed by low speed centrifugation and the parasite lysate was bound to beads overnight at 4^{o}C. Beads were washed with PBS and bound proteins were eluted directly in rehydration buffer (9.8 M urea, 0.5%

CHAPS) for 2-D gel analysis, or non-reducing protein loading buffer supplemented with 1% SDS and phosphatase inhibitors for 1D gel analysis.

^{32}P labeling of parasites

Extracellular parasites (~1-2 x 10^8) were harvested and equilibrated in labeling media (phosphate-free DMEM (Invitrogen, Carlsbad, CA) supplemented with 1% fetal bovine serum) for 1 hr at 37^{o}C. For the labeling reaction, parasites were resuspended in 450 µl labeling media to which 50 µl of 0.5 mCi orthophosphate (PerkinElmer, Boston, MA) was added. Parasites were labeled with ^{32}P for 4 hr at 37^{o}C. Following the labeling reaction, samples were maintained at 4^{o}C. Free label was removed with two PBS washes containing 50 mM sodium fluoride, and parasites were resuspended in lysis buffer (8M urea, 4% CHAPS, 40 mM Tris base) supplemented with 50 mM sodium fluoride, 10 mM sodium orthovanadate, 5 mM EDTA, protease inhibitor cocktail, and Dnase1. Following a 15 min incubation at 4^{o}C, parasite lysates were briefly centrifuged to remove insoluble material, and samples were prepared for 2-D gel electrophoresis using the 2-D clean-up kit (GE Healthcare, Piscataway, NJ) as described in the 2-D gel electrophoresis section of the methods.

Following 2-D gel electrophoresis, the proteins were transferred to nitrocellulose membranes and blots were stained with Sypro Ruby blot stain (Invitrogen, Carlsbad, CA) to visualize the total protein levels. ^{32}P labeled proteins were detected by exposing the blot to phosphorimager plates (Fuji Film Medical Systems; Stamford, CT), and after 3 weeks the plates were analyzed for autoradiography using a FLA-5000 phosphorimager (Fuji Film Medical Systems; Stamford, CT). To identify the location of ADF on the

autoradiograph, the same blot was immunoblotted with rabbit anti-TgADF antibody and developed as described above. The autoradiograph and western blot images were superimposed using Adobe Photoshop (Adobe Systems Inc., San Jose, CA) to align the edges of the blot in both images, to determine if any ^{32}P spots corresponded to ADF.

2-D gel electrophoresis

Following detergent extraction of parasite lysates, samples were prepared for 2-D gel electrophoresis using the 2-D clean-up kit (GE Healthcare, Piscataway, NJ) according to manufacturer's recommendations. Samples were loaded via rehydration loading onto 7 cm Immobiline Drystrip gels, pH 3-11(NL) or pH 6-11 (GE Healthcare, Piscataway, NJ) as indicated, and isoelectric focusing was carried out using the Ettan IPGphor II Isoelectric Focusing System (GE Healthcare, Piscataway, NJ). Proteins were separated in the second dimension by 15% SDS-PAGE.

Mass spectrometry

Parasites (1×10^9 cells per immunoprecipitation) were harvested and extracted in RIPA buffer supplemented with 50 mM sodium fluoride, 10 mM sodium orthovanadate, 5 mM EDTA, protease inhibitor cocktail, and Dnase1, for 6 hr at 4^oC. After briefly centrifuging to remove insoluble material, parasite lysates were immunoprecipitated overnight as described above. Beads were washed once with RIPA buffer and three times with PBS before proteins were eluted in non-reducing tricine gel loading buffer. Immunoprecipitated ADF was resolved on a 10-20% tricine gel, and an unstained molecular marker was run in parallel to quantify the amount of protein in the ADF bands.

Total protein was stained with the Sypro Ruby gel stain and the protein bands quantitated using a FLA-5000 phosphorimager (Fuji Film Medical Systems; Stamford, CT). The three putative ADF bands were excised separately from two separate immunoprecipitations, and submitted for in-gel trypsin digestion after which the corresponding bands from the two immunoprecipitations were pooled and analyzed by liquid chromatography-tandem mass spectrometry (Proteomics & Mass Spectrometry Facility, Washington University in St. Louis, St. Louis, MO).

RESULTS / DISCUSSION

Cloning, expression and generation of a specific antibody to *T. gondii* ADF.

To study the function and activity of ADF in *T. gondii*, we cloned recombinant *T. gondii* ADF (TgADF) with a C-terminal histidine tag (Figure 1A) to allow for recombinant protein purification. Expression of recombinant TgADF (rTgADF) in *Escherichia coli* BL21 cells resulted in a largely soluble protein that was purified from crude bacterial lysate using nickel resin (Figure 1B). Purification under native conditions yielded a protein fraction that, by Coomassie blue detection, consisted of a predominant species of the expected molecular weight of TgADF (~15 kDa) (arrow), with two minor contaminating bands (Figure 1B). This protein was used as an antigen to generate a specific antibody towards TgADF. A potent polyclonal rabbit antibody was produced that reacted with the original rTgADF antigen by western blot (Figure 1C), and detected the corresponding ADF band in parasite lysate, but importantly, did not cross-react with ADF/cofilin proteins found in host cells (Figure 1C). Although originally expressed with a C-terminal histidine tag, we have subsequently determined that rTgADF has increased purity, stability and protein activity when expressed with an N-terminal histidine tag (data not shown). Therefore, in subsequent biochemical assays defining the activities of TgADF (Chapter 3) the N-terminally tagged protein was used.

The experiments shown above demonstrated that we could express and purify soluble rTgADF protein, and a similar purification scheme was used to obtain protein for the biochemical experiments that follow. Generation of a specific antibody to TgADF

provided an important tool to specifically detect ADF in parasites and was used throughout this thesis to monitor TgADF activity *in vivo*.

Localization of ADF and actin at different stages of the *T. gondii* tachyzoite life cycle.

AC proteins have been shown to play key roles in regulating actin filament turnover in motile cells, and typically localize to the leading edge of motile cells or other areas of high actin turnover (Bamburg and Bray, 1987; Yonezawa *et al.*, 1987; Aizawa *et al.*, 1997). We wanted to compare ADF and actin localization in motile vs. non-motile *T. gondii* tachyzoites to determine if TgADF might have a role in filament turnover.

In intracellular parasites (Figure 2, top panel), which is a non-motile stage, both ADF (green) and actin (red) were diffusely localized throughout the parasite cytosol. This distribution is more clearly indicated by the profile analysis (Figure 2, top panel, right), which measures the pixel intensity across the line drawn in the micrographs, and depicts the fairly even distribution of ADF and actin in the cytoplasm of intracellular parasites. In extracellular parasites (Figure 2, middle panel), that were kept non-motile by maintaining the parasites at 18^{o}C to prevent secretion of surface adhesins required for gliding motility, a similar diffuse cytoplasmic localization of both ADF and actin was observed. In contrast, in motile parasites (Figure 2, bottom panel), which were distinguished by the deposition of a membranous trail that is left behind as the parasite glides across BSA-coated coverslips at 37^{o}C, both ADF and actin were localized at the parasite periphery in almost all motile parasites. The presence of both proteins in parasite trails suggested that they were localized underneath the parasite's plasma membrane during gliding motility.

Thus, similar to other eukaryotic systems, TgADF appears to relocalize to regions of high filament turnover, and this is suggestive that TgADF plays a role in regulating actin filament turnover during gliding motility.

Quantitation of TgADF concentration in *T. gondii*

Based on the immunolocalization data, ADF and actin appeared to co-localize in the parasite even when both proteins were dispersed throughout the cell. The cellular concentration of actin in *T. gondii* is estimated to be ~ 45 μM (updated from an earlier calculation of 10 μM, cited as unpublished data in (Sahoo *et al.*, 2006)). To determine the cellular concentration of TgADF we performed quantitative western blotting of serial dilutions of *T. gondii* tachyzoites with the TgADF antibody (Figure 3A), and compared the signal to a standard curve generated using recombinant TgADF protein (Figure 3B). Our calculations indicate that there is approximately 260 ng of ADF in a single parasite. Using the estimated volume for a tachyzoite detailed in the methods section, the molar concentration of ADF in a single parasite was estimated to be approximately 37 μM. Our estimates indicate that ADF and actin are present in parasites at a ratio of approximately 0.8:1, or roughly equimolar ratios.

The similar concentrations of TgADF and TgACT in the parasite cytoplasm initially surprised us, since filament severing, thought to be the predominant activity of AC proteins, is reported to occur optimally at AC concentrations that are far below the dissociation equilibrium constant (Andrianantoandro and Pollard, 2006). However the biochemical analysis of TgADF activities presented in Chapter 3, reveals that TgADF belongs to a small subset of AC proteins that have potent actin monomer sequestering

activity and function in cellular environments where a large proportion of the actin is monomeric. In light of this finding, the almost equimolar ratio of ADF and actin in *T. gondii*, and the moderate affinity of TgADF for TgACT (0.8 μM, reported in Chapter 3), suggests that TgADF may play an important role in sequestering monomeric actin in the parasite and maintaining high G-actin concentrations.

Disassembly of TgACT filaments by TgADF as observed by fluorescence microscopy.

AC proteins are typically known for their essential function in regulating actin filament turnover. Previous work using actin sedimentation assays, has shown that recombinant TgADF causes the net disassembly of rabbit actin filaments (Allen *et al.*, 1997). However an interaction between TgADF and TgACT had not been demonstrated. This was partly due to the difficulty in detecting TgACT filaments even with recombinant protein, since in contrast to higher eukaryotic actins, the addition of polymerization buffer to TgACT does not induce the formation of long stable actin filaments (Sahoo *et al.*, 2006). A research associate in the Sibley lab, Keliang Tang, determined that stable TgACT filaments were formed when polymerized in the presence of equimolar unlabeled phalloidin (an actin stabilizing agent), and filaments could be visualized by fluorescence microscopy with the addition of low levels of fluorescently conjugated phalloidin. Using this assay, we directly observed the disassembly of TgACT filaments in the presence of TgADF.

When TgACT was polymerized in the presence of equimolar phalloidin, long, stable TgACT filaments and bundles were detected (Figure 4A, left). After incubation

with 10-fold molar excess TgADF for ~20 min (Figure 4A, middle), a significant decrease in the number of TgACT filaments and bundles was observed, and after a 1 hr incubation with TgADF (Figure 4A, right), there was a striking absence of filaments. This demonstrated for the first time that TgADF could interact with and disassemble TgACT filaments in a time-dependent manner.

The N-terminus of AC proteins is highly conserved amongst family members and has been shown to be required for interacting with both F- and G-actin (Lappalainen *et al.*, 1997). In many systems AC protein activity is negatively regulated by phosphorylation on a highly conserved serine 3 residue (Bamburg, 1999). I have shown that the serine 3 residue is important for TgADF interactions with rabbit actin using actin sedimentation assays (Chapter 3). To determine if this site was also important for interactions with TgACT, we polymerized TgACT in the presence of TgADF harboring the S3E mutation, and observed its effect on the activity of TgADF. In the absence of TgADF, TgACT generated long stable filaments and bundles (Figure 4B, left), whereas when polymerized in the presence of wildtype TgADF, only short actin stubs or aggregates were detected (Figure 4B, middle). In contrast, when TgACT was polymerized in the presence of S3E TgADF (Figure 4B, right), long stable filaments and bundles were observed that were indistinguishable from TgACT polymerized alone (Figure 4B, left). These data suggested that the S3E TgADF protein was unable to interact with TgACT, and that the N-terminus of TgADF, similar to its interaction with rabbit actin, was important for interactions with TgACT.

Since phalloidin is known to antagonize the activity of AC proteins and has previously been shown to inhibit the disassembly of actin filaments by cofilin (Yonezawa

et al., 1988; Hayden *et al.*, 1993), it is interesting that TgADF was able to completely disassemble TgACT filaments that had been stabilized with equimolar phalloidin. This activity may have been due to the high concentrations of TgADF that were used in the experiment (10-fold molar excess TgADF), the relatively long time-scale of the experiment, a difference in the nature of phalloidin stabilized TgACT filaments so that they are more readily disassembled than conventional actin filaments stabilized with phalloidin, or a unique feature of how TgADF interacts with TgACT filaments. To begin to address these questions, future experiments should be performed using a control AC protein such as *S. pombe cofilin* in parallel, to see if phalloidin-stabilized TgACT is more susceptible to disassembly than what has previously been observed with other phalloidin-stabilized actins, or if there is something different about the TgADF-TgACT interaction that enhances the ability of TgADF to diassemble stabilized TgACT filaments. Additionally it will be important to titrate the amount of TgADF used in these assays to determine if such high concentrations of TgADF are necessary to observe activity.

Overall these experiments demonstrated that TgADF is able to disassemble TgACT filaments in a time-dependent manner, and that the N-terminus of TgADF harboring the conserved serine 3 residue is important for TgADF interactions with TgACT.

Expression of serine 3 mutant ADF alleles in *T. gondii*.

While replacement of the serine 3 residue in ADF with a bulky negatively charged residue such as glutamic acid inhibited the interaction between TgADF and actin *in vitro*, mutation to a small similarly charged residue such as alanine or cysteine is predicted to

have little effect on the protein's *in vitro* activity. However, if phosphorylation of the serine 3 residue is necessary to regulate ADF activity *in vivo*, the absence of serine at this residue might have striking consequences *in vivo,* resulting from the protein being constitutively active and constantly interacting with actin. This suggests that expression of an S3A ADF mutant allele might have a dominant effect *in vivo* even when expressed in the presence of the endogenous wildtype allele, whereas an S3E mutant allele is expected to have no effect.

To test if phosphorylation is important for regulating TgADF activity *in vivo*, we introduced a second ADF allele into parasites, that was either wildtype or harbored the S3A or S3E point mutation, and isolated stable parasite clones. Western blot analysis confirmed expression of the second allele (Figure 5A), and clones that had the highest relative expression of the tagged protein to the endogenous protein were chosen for further experiments (Figure 5A). The ability to invade host cell monolayers was compared amongst the strains using the two-color invasion assay (Buguliskis *et al.*, 2010), which is based on the differential staining of extracellular and intracellular parasites. No differences in invasion were observed between parasites expressing the wildtype, S3A, or S3E ADF alleles (Figure 5B). Clones were also compared using plaque assays and gliding assays (data not shown). Similar to the invasion assay, no differences were observed between the strains, suggesting that the S3A ADF allele did not have a dominant effect over the endogenous wildtype ADF allele.

Since it is possible that expressing high levels of a mutant allele with a strong dominant negative effect may proclude obtaining stable clones of these parasites, transiently transfected parasite populations were also assayed for gliding and invasion

phenotypes. No differences in behavior were observed even in parasites that appeared to be strongly expressing the S3A ADF allele by immunofluorescence analysis (data not shown).

While the S3A ADF allele did not have a dominant effect in these experiments, this may be due to the low expression of the mutant allele relative to the endogenous allele. At this low expression level, there may have not been enough mutant ADF protein to perturb the system. To overcome this limitation, a stronger promoter could be used to drive expression of the tagged alleles. However, to conclusively determine whether phosphorylation is important for regulating ADF activity *in vivo* it will be necessary to determine the effect of the S3A TgADF allele in the absence of the wildtype TgADF allele. This could be examined in future experiments using the TgADF conditional knockout that is generated in Chapter 4.

Post-translational modifications of TgADF *in vivo*.

Since the activity of many AC proteins is regulated by phosphorylation (Bamburg, 1999), we wanted to determine if we could detect phosphorylated TgADF in parasites. To do this, ADF was isolated from parasites and its composition analyzed by 2-D gel electrophoresis. ADF immunoprecipitated from *T. gondii* resolved as multiple spots on 2-D gels, suggesting that TgADF is post-translationally modified *in vivo* (Figure 6A). A variety of approaches were used to determine if any of the spots represented a phosphorylated form of TgADF. These approaches included phosphatase treatment of the blot or the protein (Morgan *et al.*, 1993; Zhuo *et al.*, 1993), blotting with phosphocofilin antibodies (Melendez-Vasquez *et al.*, 2004; Shaw *et al.*, 2004), staining with ProQ

diamond phosphoprotein stain (Jacob and Turck, 2008) and analysis of spots by mass spectrometry (Tomaiuolo *et al.*, 2009). The above methods failed to resolve whether or not TgADF was phosphorylated, and this was either due to technical difficulties, or limits to the sensitivity of the method. The lack of a phosphorylated *T. gondii* control protein that could be treated in parallel also limited our ability to determine the validity of some of the assays.

To maximize the sensitivity of phosphoprotein detection, we labeled extracellular parasites with ^{32}P orthophosphate (Figure 6B). Following labeling, parasite lysates were resolved by 2-D gel electrophoresis and transferred to nitrocellulose so that the location of ADF on the blot could be identified by western blotting for TgADF. Due to the weak ^{32}P signal in the lower molecular weight range, the autoradiograph was exposed for 3 weeks. Overlaying the autoradiograph (Figure 6B, left) with the ADF western of the same blot (Figure 6B, right) revealed one ADF spot (Figure 6B, circle) that co-migrated with a ^{32}P spot (Figure 6B, circle). This was observed in 2 independent experiments. To determine whether the highlighted ^{32}P spot represented a phosphorylated form of TgADF, or if it represented the co-migration of another phosphorylated protein with ADF, we attempted to immunoprecipitate ADF from ^{32}P labeled parasites. Unfortunately the low levels of radiation detected on the blot after transfer of the proteins to nitrocellulose, and the minimal amount of ^{32}P detected after blot exposure, rendered these experiments inconclusive. While the low levels of ^{32}P may indicate the lack of ADF phosphorylation, sypro ruby staining of the blots also suggested that the amount of ADF immunoprecipitated was limiting in these experiments. Therefore, our conclusion from the ^{32}P labeling experiments is that one ADF spot migrates identically with a ^{32}P spot, but

it is unclear if this ^{32}P spot represents a phosphorylated form of TgADF, or an unrelated comigrating protein.

To identify other modifications that TgADF might be subjected to, ADF immunoprecipitated from parasites was resolved using 1-D tris-tricine gels (Figure 6C) and analyzed by mass spectrometry. TgADF resolved as 3 separate bands on tricine gels (Figure 6C, ADFIP A and B, arrows). The indicated bands, which contained between 120-220 ng or 8-15 picomoles of protein per band, were excised from the gel and the corresponding bands from the two immunoprecipitations were pooled and submitted for liquid chromatography-electrospray ionization tandem mass spectrometry analysis. However, due to the low signal to noise ratio, TgADF was only identified in the top band (which combined, contained 26 picomoles of ADF), and there was insufficient protein for the analysis of post-translational modifications.

Although our data indicates that TgADF is post-translationally modified *in vivo*, attempts to identify the modifications were unsuccessful. We determined that one of the TgADF isoforms migrates identically to a ^{32}P labeled phosphoprotein, but it is unclear if this ^{32}P spot represents a phosphorylated form of TgADF, or the co-migration of TgADF with another phosphorylated protein. Apart from phosphorylation on the conserved serine 3 residue, some AC proteins are acetylated on the penultimate residue after cleavage of the initiator methionine (Bamburg, 1999). Such modifications may also occur in TgADF, or the different forms may represent novel modifications.

Improved mass spectrometry methods with increased sensitivity that are now available, such as the Orbitrap mass spectrometer (Hu *et al.*, 2005) may be better able to resolve the different TgADF isoforms. Fragmentation analysis may resolve whether they

represent typical modifications undergone by AC proteins, or if TgADF undergoes novel modifications that may be specific to its activity in the parasite. Further work would need to establish if these modifications had a role in regulating TgADF activity or localization. Additionally, if this line of investigation is to be pursued, scaling up in the immunoprecipitation procedure and in the 2-D gel format will be required to isolate sufficient material for analysis.

In yeast and *Dictyostelium*, AC activity is not thought to be regulated by phosphorylation (Lappalainen *et al.*, 1997; Bamburg, 1999), and while it is unknown exactly how activity is regulated in these organisms, there is some data to suggest a role for AC protein inactivation by phosphoinositide binding at the plasma membrane (Ojala *et al.*, 2001; Gorbatyuk *et al.*, 2006). This could be an alternative mechanism by which TgADF activity is regulated in the parasite, in addition to regulation by other actin binding proteins.

Summary

The work presented in this chapter generated the tools and reagents for the further study of the biochemical activity and function of TgADF in the parasite. Our preliminary data indicated that TgADF was relocalized to the parasite plasma membrane during gliding motility, a finding that is consistent with the relocalization of AC proteins to regions of high filament turnover in the cell. Using direct observation by fluorescence microscopy we showed that TgADF was able to disassemble TgACT filaments in a time-dependent manner, and that the N-terminus of TgADF was important for this interaction. Finally we showed that TgADF undergoes post-translational modification *in vivo*, although the

nature and importance of these alterations remains unknown. Overall the data presented in this first chapter generated the reagents for many of the experiments to come, and the preliminary characterization of TgADF described here provided a foundation on which the remainder of this thesis was built.

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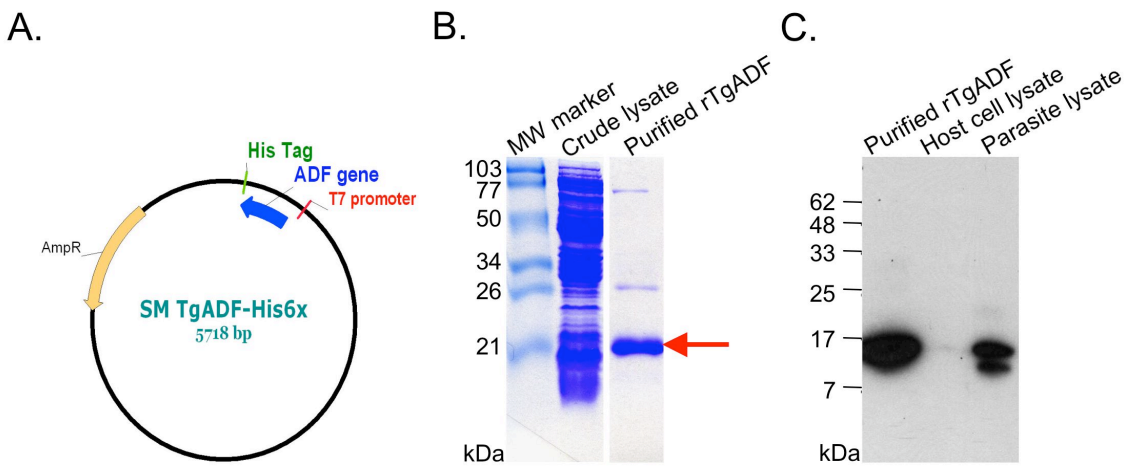

Figure 1. Cloning, expression and generation of a specific antibody to TgADF. A) Plasmid map of recombinant TgADF (rTgADF). The TgADF gene was cloned with a C-terminal His_{6x} tag, under the T7 promoter. B) Coomassie blue stained SDS-PAGE depicting the purification of rTgADF protein. rTgADF was expressed in BL21 cells and purified over nickel resin. Crude bacterial lysate and purified rTgADF protein were resolved by 15% SDS-PAGE. Arrow indicates the major species purified runs at ~15kDa, the expected M_r of TgADF. C) Western blot showing the specificity of the TgADF antibody. rTgADF protein was used to generate an anti-TgADF antibody. The specificity of the antibody was tested against recombinant TgADF protein, host cell lysate and parasite lysate resolved by 15% SDS-PAGE.

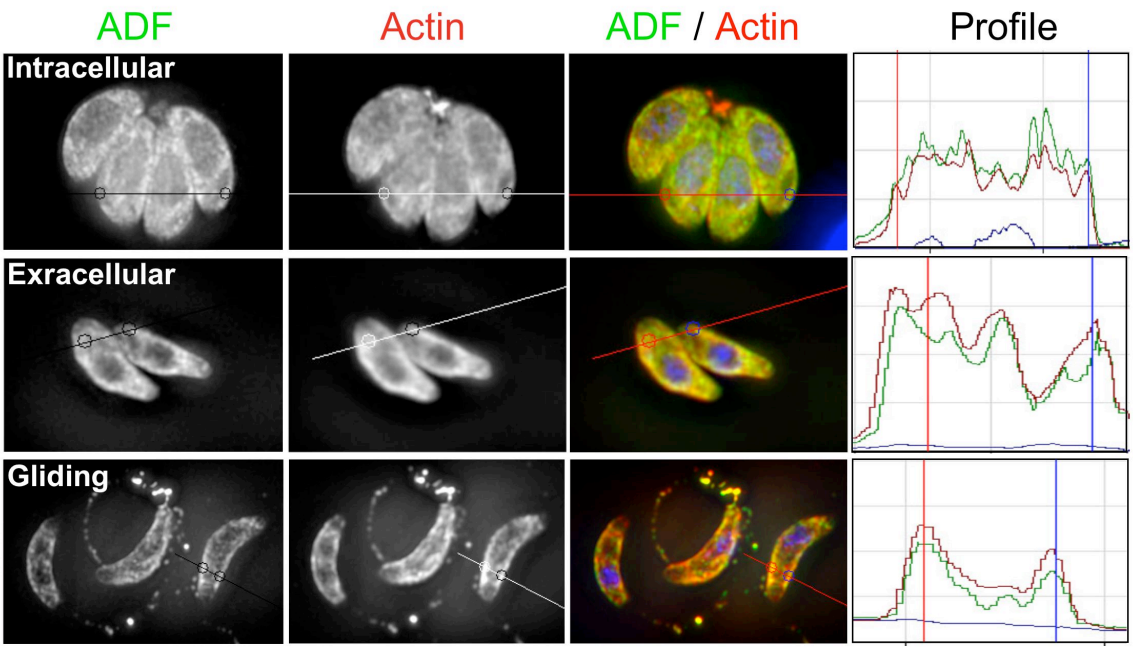

Figure 2. Localization of ADF and actin at different stages of the *T. gondii* tachyzoite life cycle. Immunofluorescence micrographs depicting the localization of ADF and actin in intracellular (top), extracellular (middle), and gliding (bottom) parasites. ADF (green) and actin (red) were detected using rat anti-TgADF and rabbit anti-TgACT antibodies, followed by Alexa conjugated secondary antibodies. Extracellular parasites were kept non-motile by keeping parasites at 18^{o}C during the assay to prevent secretion of surface adhesins required for gliding motility. Gliding parasites were allowed to glide on BSA-coated coverslips at 37^{o}C for 15 min. Profile analysis depicts the pixel intensity across the line drawn through the parasites, with the red and blue circles in the merged image corresponding to the red and blue line in the profile. The color code of the histograms matches the stained proteins.

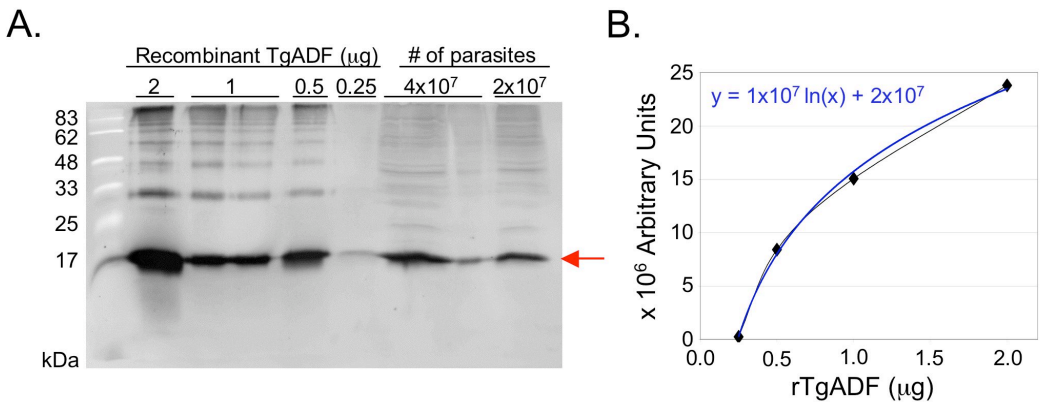

Figure 3. Quantitation of TgADF concentration in *T. gondii.* A) Quantitative western blot to determine the amount of ADF in parasites. Parasite lysates were resolved by 15% SDS-PAGE and probed with a rabbit anti-TgADF antibody to determine the amount of ADF in parasites relative to a standard curve of purified TgADF protein. Arrow denotes the ADF band. B) Standard curve to calculate the amount of ADF in parasites. The ADF bands for the rTgADF protein in A) were quantitated by phosphorimager analysis and used to generate a standard curve that was subsequently used to calculate the concentration of ADF in parasites. The equation of the best fit line to the standard curve is depicted on the graph.

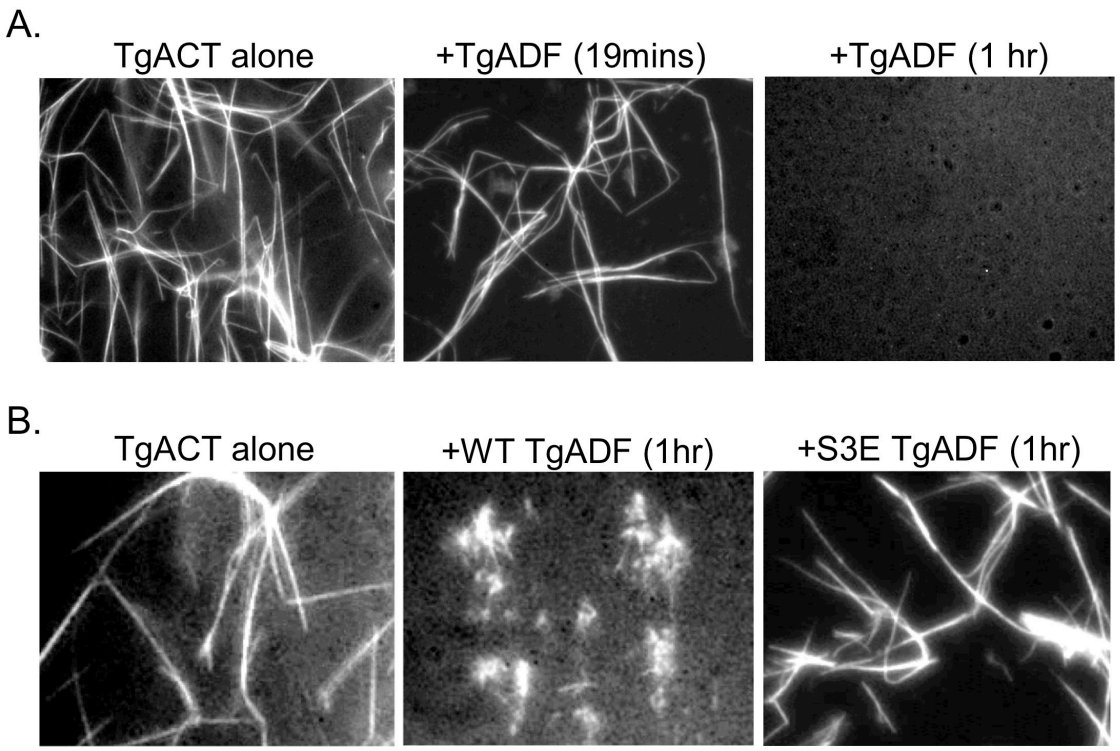

Figure 4. Disassembly of TgACT filaments by TgADF as observed by fluorescence microscopy. A) Disassembly of TgACT filaments by TgADF followed over time. Fluorescence micrographs of recombinant TgACT filaments were taken before or after incubation with 10-fold molar excess TgADF at the indicated time. Recombinant TgACT (5 μM) was polymerized with equimolar unlabeled phalloidin and a low-level of Alexa Fluor 488-labeled phalloidin (0.33 μM) for visualization. B) Effect of the S3E mutation on TgADF interaction with TgACT filaments. Fluorescent micrographs of TgACT filaments were taken after 1 hr incubation with wild-type (WT) TgADF, or a mutant TgADF in which the serine 3 residue had been mutated to glutamic acid (S3E TgADF). TgACT was polymerized and visualized as described in A) and in the presence of 10:1 molar excess TgADF.

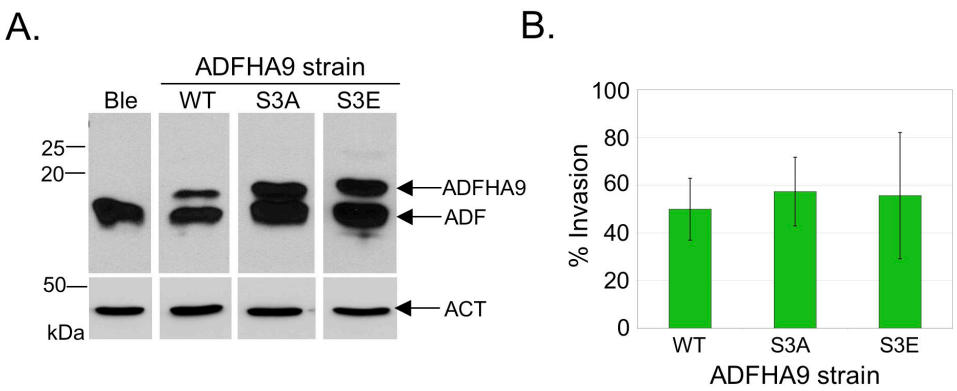

Figure 5. Expression of serine 3 mutant ADF alleles in *T. gondii*. A) Western blot analysis of parasites expressing ADFHA9 transgenes that have point mutations at the serine 3 residue. Expression of the ADF transgenes was detected by probing parasite lysates with a rabbit anti-TgADF antibody that detected both endogenous ADF and ADFHA9 proteins. Detection of actin (ACT) with the rabbit anti-*T. gondii* actin antibody served as a loading control. Ble, represents a control clone expressing the selection marker used to introduce the transgenes into parasites. WT, S3A and S3E indicate whether the transgene contained the wild-type ADF allele, ADF with a serine to alanine mutation at residue 3, or ADF with a serine to glutamic acid mutation at residue 3, respectively. B) Comparison of invasion efficiencies in parasites expressing the serine 3 mutant ADF alleles. Parasite invasion of host cell monolayers in a 20 min time-period was detected using the two-colour invasion assay, where intracellular and extracellular parasites are differentially stained against a parasite surface antigen. Data are plotted as the number of parasites invaded out of the total number of parasites counted. N=1, 12 fields counted per strain. Values represent mean ± SD.

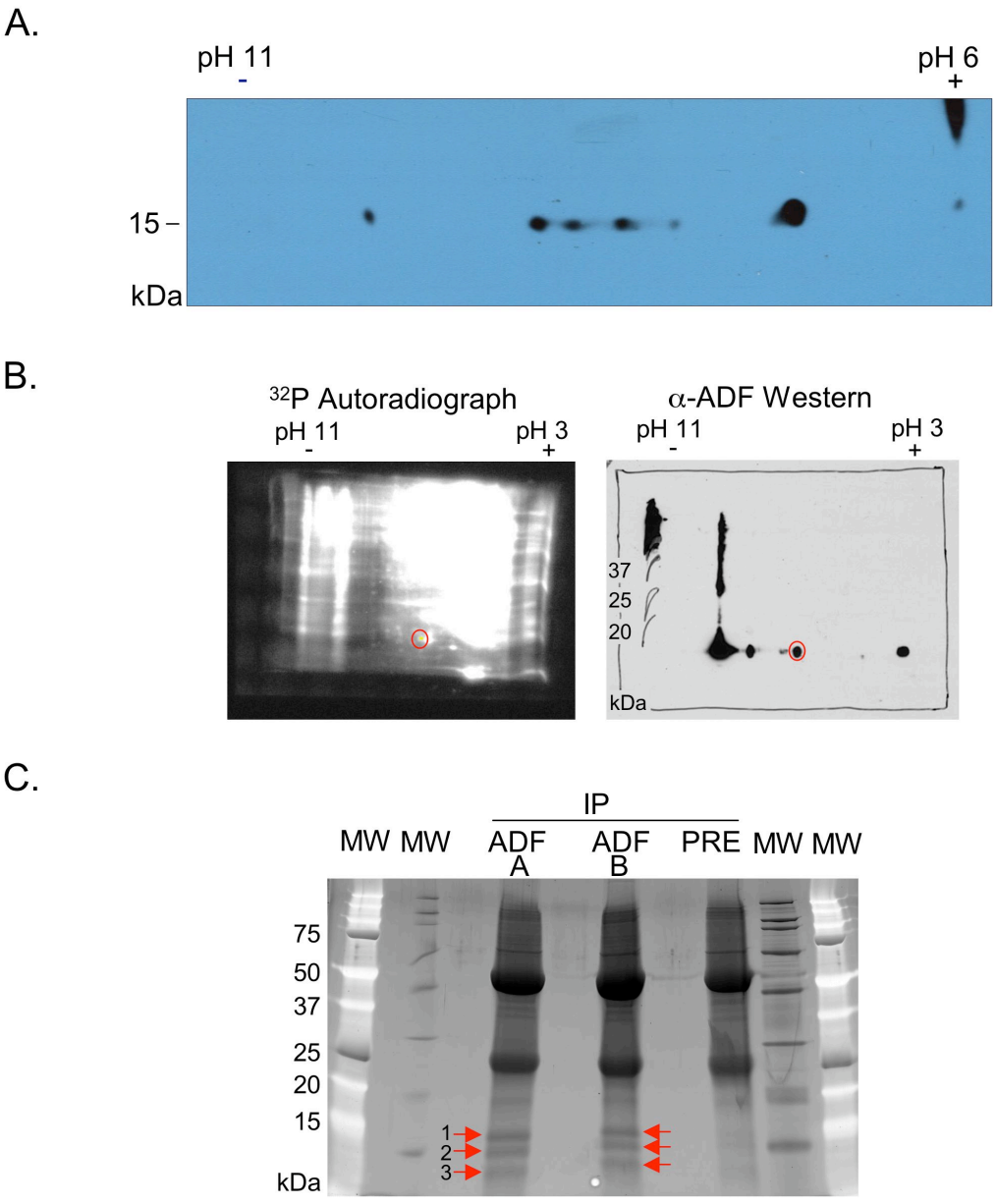

Figure 6. Post-translational modifications of TgADF *in vivo*. A) Western blot analysis of ADF immunoprecipitated from extracellular parasites and resolved by 2D gel electrophoresis. Immunoprecipitated ADF was resolved on Immobiline Drystrip pH 6-11 in the first dimension, and by 15% SDS-PAGE in the second dimension. ADF was detected with the rabbit anti-TgADF antibody. B) 2D gel analysis of ^{32}P labeled extracellular parasites. Western blot (right) and ^{32}P autoradiograph (left) of the same gel

representing extracellular parasites that were labeled with orthophosphate and resolved by 2D gel electrophoresis. The two images were overlayed to determine the location of ADF on the autoradiograph (not shown). Extracellular parasites were labeled with 0.5 mCi orthophosphate for 4 hr and parasite proteins were isoelectrically focused on Immobiline Drystrip pH 3-11 (NL) and resolved in the second dimension by 15% SDS-PAGE. After transfer to nitrocellulose and exposure of the autoradiograph, ADF was detected using the rabbit anti-TgADF antibody. The ^{32}P spot that co-migrates with TgADF was colored yellow for easier visualization and the spot of interest is outlined with a red circle on both the western and the autoradiograph. C) Immunoprecipitated ADF from extracellular parasites resolved using tris-tricine gel electrophoresis. Sypro Ruby staining of immunoprecipitated ADF (ADFIP) resolved on a 10-20% tris-tricine gel revealed 3 bands (arrows) not detected in the control immunoprecipitation (PRE-IP). ADF was immunoprecipitated with a rabbit anti-TgADF antibody and the control immunoprecipitation was performed with pre-immune serum (PRE-IP). MW refers to the molecular weight marker.

Table S1. Primer sequences used to generate plasmid constructs

Primer	Use	Forward (5'->3')	Reverse (5'->3')
ADF	To amplify *ADF* from cDNA	GGGAATCCATATGGC GTCCGGAATGGGTGT TG	CGGCCTCGAGCGCG AGGGGTGCGAGGTC GC
ADFHA9	To amplify *ADF* with a C-terminal HA9 tag	GGGAAGCTTATGGCG TCCGGAATG GGTGTTGACG	CGGTTAATTAAGCGT AATCTGGGA CGTCGTATGGGT ACGCGAGGGGTGCG AGGTCGCCC
ADFPromoter	To amplify the 1kb genomic sequence upstream of *TgADF*	GCGAAGCTTCGGCAG GGCAGCGAAGAGG	AGCACCCCGGAATT CGTCCG
S3A ADFHA9	To make point mutation in *ADF*	CCAGTCAAGATGGCG GCCGGAATGGGTGTT G	CAACACCCATTCCGG CCGCCATCTTGACTG G
S3E ADFHA9	To make point mutation in *ADF*	CTCCAGTCAAGATGG CGGAGGGAATGGGTG TTGACG	CGTCAACACCCATTC CCTCCGCCATCTTGA CTGGAG

Chapter 3

Toxoplasma gondii Actin Depolymerizing Factor acts primarily to sequester G-actin

PREFACE

This first complete draft of this chapter was written by Simren Mehta. Comments from L. David Sibley and reviewers were incorporated into the final version presented here.

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ABSTRACT

Toxoplasma gondii is a protozoan parasite belonging to the phylum Apicomplexa. Parasites in this phylum utilize a unique process of motility termed gliding, which is dependent on parasite actin filaments. Surprisingly, 98% of parasite actin is maintained as G-actin, suggesting that filaments are rapidly assembled and turned over. Little is known about the regulated disassembly of filaments in the Apicomplexa. In higher eukaryotes, the Actin Depolymerizing Factor/Cofilin (AC) proteins are essential regulators of actin filament turnover. ADF is one of the few actin binding proteins conserved in apicomplexan parasites. In this study we examined the mechanism by which *T. gondii* ADF (TgADF) regulates actin filament turnover.

Unlike other members of the AC family, apicomplexan ADFs lack key F-actin binding sites. Surprisingly, this promotes their enhanced disassembly of actin filaments. Restoration of the C-terminal F-actin binding site to TgADF stabilized its interaction with filaments, but reduced its net filament disassembly activity. Analysis of severing activity revealed that TgADF is a weak severing protein, requiring much higher concentrations than typical AC proteins. Investigation of TgADF's interaction with *T. gondii* actin (TgACT) revealed that TgADF disassembled short TgACT oligomers. Kinetic and steady-state polymerization assays demonstrated that TgADF has strong monomer sequestering activity, inhibiting TgACT polymerization at very low concentrations. Collectively these data indicate that TgADF promoted the efficient turnover of actin filaments via weak severing of filaments and strong sequestering of monomers. This

suggests a dual role for TgADF in maintaining high G-actin concentrations and effecting rapid filament turnover.

INTRODUCTION

Toxoplasma gondii is an obligate intracellular protozoan parasite that belongs to the phylum Apicomplexa. In addition to being a significant cause of disease in immunocompromised individuals (Joynson and Wreghitt, 2001), *T. gondii* also provides an excellent model system for other members of the phylum (such as the medically important *Plasmodium* species), due to the presence of a variety of experimental tools. Transmission of *T. gondii* typically occurs via the ingestion of tissue cysts in undercooked meat or oocysts that are shed by cats and which can contaminate water (Mead *et al.*, 1999). Once inside the host, the parasite utilizes a unique mode of motility, termed gliding, to move across epithelial barriers and migrate into deeper tissues (Barragan and Sibley, 2003). Gliding motility is conserved across the Apicomplexa, and is responsible for the parasite's invasion of host cells (Sibley, 2004). Both gliding and cell invasion depend on actin filaments within the parasite (Dobrowolski and Sibley, 1996; Wetzel *et al.*, 2003).

In contrast to higher eukaryotic cells where typically 50% of the actin is filamentous (Korn, 1978), actin in *T. gondii* is almost exclusively unpolymerized (Dobrowolski *et al.*, 1997; Wetzel *et al.*, 2003; Sahoo *et al.*, 2006). *T. gondii* has one actin allele (TgACT1)[1] that has 83% identity to vertebrate actin, and is expressed throughout the parasite's life cycle (Dobrowolski *et al.*, 1997). Recent work has shown that parasite actins are inherently unstable (Schmitz *et al.*, 2005; Schuler *et al.*, 2005a; Sahoo *et al.*, 2006), and this is likely due to key substitutions in their molecular structure (Sahoo *et al.*, 2006). Actin typically undergoes self-polymerization above a critical

concentration of 0.12 μM (Pollard *et al.*, 2000); however, the critical concentration for TgACT is surprisingly low at only 0.03 μM (Sahoo *et al.*, 2006). Despite maintaining high cellular concentrations of G-actin, filamentous actin is essential for both gliding motility and host cell invasion by *T. gondii* (Dobrowolski and Sibley, 1996). Treatment of parasites with cytochalasin D, renders parasites non-motile and unable to penetrate host cells, and genetic studies using mutants indicate that the polymerization of parasite filaments is crucial for both gliding and invasion (Dobrowolski and Sibley, 1996). Additionally, hyper-stabilizing actin filaments by treatment with jasplakinolide (JAS) (Poupel and Tardieux, 1999; Shaw and Tilney, 1999) is also toxic to the parasite. Although JAS-treated parasites move with three-fold increased speed (Wetzel *et al.*, 2003) (indicating that actin polymerization is rate-limiting for motility), the prevention of filament turnover results in the inability of parasites to pursue directional movement and invade host cells (Wetzel *et al.*, 2003). Collectively, these studies indicate that the control of parasite filament turnover is critical for productive gliding and invasion.

The unique actin dynamics observed in apicomplexan parasites suggest that actin polymerization and turnover are tightly regulated. However, in contrast to higher eukaryotes, which have many actin binding proteins to regulate the microfilament system (dos Remedios *et al.*, 2003), only a small set of actin binding proteins are conserved in the Apicomplexa (Baum *et al.*, 2006; Schüler and Matuschewski, 2006; Gordon *et al.*, 2007). This subset includes formins, profilin, capping protein, actin depolymerizing factor (ADF), cyclase-associated protein (CAP) and coronin, which represent little more than the core set of actin binding proteins required to reconstitute motility *in vitro* (Carlier *et al.*, 1997). Considering the high G-actin concentrations in apicomplexan

parasites, it is surprising that no dedicated G-actin sequestering proteins are present, such as β-thymosin found in higher eukaryotes (Nachmias, 1993).

Recent work has shed some light on to how actin filaments may be assembled in apicomplexan parasites. *Plasmodium* formins have been shown to nucleate heterologous actin polymerization *in vitro* (Baum *et al.*, 2008), and *Toxoplasma* profilin was shown to allow steady-state barbed end growth while causing depolymerization at the pointed end (Plattner *et al.*, 2008). Most recently *Plasmodium* capping protein was shown to cap heterologous actin filaments (Ganter *et al.*, 2009). Although these studies provide insight into the actin polymerization machinery in apicomplexan parasites, little is known about the regulated disassembly of parasite actin filaments.

The actin depolymerizing factor (ADF)/Cofilin (AC) family of proteins are highly conserved proteins, and are essential for increasing actin filament turnover in higher eukaryotes (Lappalainen and Drubin, 1997). AC proteins are found ubiquitously in eukaryotes, often present in multiple isoforms, and tend to have a conserved structure and function (Bamburg, 1999). The predominant mechanism by which they are thought to increase filament turnover is by severing actin filaments (Andrianantoandro and Pollard, 2006). Electron cryomicroscopy and helical reconstructions of ADF and cofilin bound to the side of actin filaments, reveals that they alter the twist of the filament by 4 – 5 ^{o}C, causing local alterations in the filament structure that are thought to lead to the fragmentation of filaments (McGough *et al.*, 1997). AC proteins also increase the rate at which monomers are released from the pointed ends of filaments (Carlier *et al.*, 1997; Maciver *et al.*, 1998).

Mutational analysis of yeast cofilin reveals several sites required for actin binding (Lappalainen *et al.*, 1997), many of which have also been confirmed in other AC proteins using mutagenesis (Moriyama *et al.*, 1992; Moriyama and Yahara, 1999; Ono *et al.*, 1999), cross-linking (Yonezawa *et al.*, 1991), peptide competition or synchrotron electron footprinting (Guan *et al.*, 2002). The actin binding sites can be classified into two types: sites required for general actin binding, and sites required exclusively for binding to filaments (29). Highly conserved residues are found at the N-terminus, which includes the putative phosphorylation site, serine 2 or 3 in eukaryotes, serine 6 in plants (Bamburg, 1999), in the long α3 helix, and in the turn connecting strand β6 and α4. These sites cluster together in the 3D-structure (Hatanaka *et al.*, 1996; Fedorov *et al.*, 1997; Leonard *et al.*, 1997) and constitute a general actin binding surface. The F-actin binding sites are less well-defined, but include a pair of basic residues at the beginning of the β5 strand (Lappalainen *et al.*, 1997; Pope *et al.*, 2000), which makes up part of the F-loop structure, and charged residues in the C-terminal α4 helix (Lappalainen *et al.*, 1997)(29) or the C-terminal tail (Ono *et al.*, 1999). The F-actin sites also appear to cluster together to form a binding surface (Fedorov *et al.*, 1997).

Recent work has shown that mutation of the F-actin binding sites in AC proteins can uncouple their severing and depolymerizing activities (Ono *et al.*, 1999; Pope *et al.*, 2000; Ono *et al.*, 2001). Point mutation of the critical basic residue K96 in the F-loop of human cofilin, leads to a loss of severing activity and increased depolymerization activity (Pope *et al.*, 2000). Interestingly, mutation of the homologous residue in *S. pombe* cofilin (R78) results in a loss of nucleating activity (Andrianantoandro and Pollard, 2006). Similarly, deletion of a charged residue in the C-tail of the *C. elegans* AC homologue,

Unc60B, results in a loss of severing activity and increased depolymerization activity (Ono *et al.*, 1999; Ono *et al.*, 2001). Collectively, these studies highlight how a reduction in the affinity for the filament can uncouple the various activities of AC proteins, and identify specific molecular features that may also influence function in other AC proteins.

Although severing is the main activity typically associated with AC proteins, they can also interact with actin in other ways, depending on the isoform, organism or cell type they are normally expressed in (Bamburg, 1999). For example, *S. pombe* cofilin was recently shown to nucleate filaments when present at high concentrations (Andrianantoandro and Pollard, 2006). In direct contrast, the *C. elegans* isoform Unc60A was found to inhibit the steady-state polymerization of actin (Yamashiro *et al.*, 2005). Thus AC proteins can influence actin dynamics in a variety of ways depending on the specific cellular context in which they function.

AC proteins are conserved in the Apicomplexa, in which they are named ADF, and most parasites possess only a single isoform, except *Plasmodium*, which has two isoforms. Preliminary characterization of *T. gondii* ADF (TgADF) (Allen *et al.*, 1997) demonstrates that it binds to G-actin, and causes the net disassembly of rabbit actin filaments *in vitro*, suggesting that TgADF accelerates filament turnover (Allen *et al.*, 1997). However the mechanism by which it does so remains unknown. Interestingly, one of the ADF homologues, PfADF1, from the related apicomplexan parasite, *Plasmodium falciparum*, does not interact with F-actin or disassemble filaments (Schuler *et al.*, 2005b). Instead, PfADF1 slightly enhances nucleotide exchange on G-actin, in contrast to the typical inhibition of nucleotide exchange caused by AC proteins (Nishida, 1985; Blanchoin and Pollard, 1998), suggesting an alternative role for ADF1 in these parasites.

More recently, ADF from the apicomplexan parasite *Eimeria tenella* was reported to be transcriptionally upregulated in the motile stages of the parasite, but little is known about the biochemical activity of this protein (Xu *et al.*, 2008).

To determine how TgADF accelerates actin filament turnover we purified recombinant TgADF and examined its interactions with both heterologous actin and *T. gondii* actin in a variety of biochemical assays. We determined that the *in vitro* properties of TgADF are related to specific features in the molecular structure, and are particularly well-suited to control the unique actin dynamics found in apicomplexan parasites. As other apicomplexan ADFs share the same molecular features, it is likely that the properties observed here are conserved in the phylum.

MATERIALS AND METHODS

Secondary-structure based sequence alignment and homology modeling of TgADF structure

A multiple sequence alignment including *T. gondii* ADF (TgADF) and other ADF/Cofilin (AC) proteins described below, was generated using Clustal X (Larkin *et al.*, 2007) with the following parameters: gap opening = 15.00, gap extension = 0.30, delay divergent sequence = 25%. The following protein sequences (with the Genbank sequence ID no.) were retrieved from NCBI and used in the alignment: AtADF1: *Arabidopsis thaliana* ADF1 (AAC72407), AcActophorin: *Acanthamoeba castellani* actophorin (AAA02909), ScCOF: *Saccharomyces cerevisiae* cofilin (AAA13256), SpCOF: *Schizosaccharomyces pombe* cofilin (CAB11258), CeUnc60A: *Caenorhabditis elegans* Unc60A (AAL02461), PfADF2: *Plasmodium falciparum* ADF2 (NP705497), HsADF: *Homo sapiens* ADF (AAB28361), PfADF1: *P. falciparum* ADF1 (NP703379), TgADF: *Toxoplasma gondii* ADF (AAC47717). The structure of TgADF was modeled onto the known crystal structure of *A. castellani* actophorin (Protein Data Bank code 1ahq) by submitting the sequence of TgADF to the Swiss Protein Data Bank. This structural model was used to subsequently manually adjust the sequence alignment of TgADF. Other sequences in the AC alignment were also manually adjusted based on the secondary structure alignment of Bowman *et al*. (Bowman *et al.*, 2000).

Proteins

Lyophilized rabbit skeletal muscle actin was obtained from Cytoskeleton (Denver, CO)

and reconstituted according to manufacturer's recommendation, while recombinant *T. gondii* actin was purified as previously described (Sahoo *et al.*, 2006). TgADF was amplified from the RH strain *T. gondii* cDNA using specific primers (Table S1), and cloned into the pET16b+ vector (Novagen, Darmstadt, Germany) at the Nde1-BamH1 site which resulted in the addition of an N-terminal His_{10X} tag. *S. pombe* cofilin (SpCOF) and *A. castellani* actophorin were digested from separate plasmids made in the pMW172 background (Quirk *et al.*, 1993; Chan *et al.*, 2009) (kindly provided by Thomas Pollard, Yale University, New Haven, CT), and cloned into the NdeI - BamHI site of pET16b+. Proteins were purified using Ni-NTA agarose according to manufacturer's recommendation (Invitrogen, Carlsbad, CA). All purified proteins were stored in G-buffer (5 mM Tris-HCl pH 8.0, 0.2 mM $CaCl_2$, 0.2 mM ATP, 0.5 mM DTT). Point mutations were introduced into pET16b+/TgADF (Table S1) using specific primers (Table S1) and the QuikChange mutagenesis kit (Stratagene, La Jolla, CA), and the sequence was verified by DNA sequencing. TgADF containing the *S. pombe* cofilin C-terminus (ADF-t) was constructed using PCR amplification with specific primers (Table S1) to add sequence encoding the last 7 amino acids (LEKVTRK) of *S. pombe* cofilin to TgADF. ADF-t was cloned into the NdeI – BamHI site of the pET16b+ expression vector, and the protein was expressed and purified as described above.

Actin Sedimentation Assays

Rabbit actin (10 μM) was polymerized by the addition of $1/10^{th}$ volume of 10X F-buffer (500 mM KCl, 20 mM $MgCl_2$, 10 mM ATP) for 30 min before the addition of ADF/Cofilin (AC) proteins. Incubation with AC proteins (0-20 μM) was carried out for 1

h at room temperature and filaments were separated by ultracentrifugation at 100,000 × g for 1 h (TL100 rotor, Beckman Optima TL ultracentrifuge, Beckman Coulter, Fullerton, CA). Pellet fractions were washed once with F-buffer to remove traces of the soluble fraction. Proteins were precipitated from the soluble fractions using acetone, and equivalent amounts of pellet and supernatant fractions were resolved by 15% SDS-PAGE. Quantitation of protein in the pellet and supernatant fractions was done by phosphorimager analysis of Sypro-Ruby stained gels using a FLA-5000 phosphorimager (Fuji Film Medical Systems, Stamford, CT). For examining the effect of pH on activity, TgADF and actin were incubated in 1X F-buffer maintained at either pH 8.2 or pH 6.8 (with the addition of 10 mM PIPES). For sedimentation assays with *T. gondii* actin, 5 μM actin was polymerized with the addition of 10X F-buffer in the presence or absence of 5 μM phalloidin (Molecular Probes, Eugene, OR). Following incubation with two-fold molar excess TgADF, samples were centrifuged at either 100,000 × g or 350,000 × g and processed as described above. Data are presented as the percent actin (of total), that pelleted after ultracentrifugation at the indicated speed. All average results are presented as mean ± S.E.M. Unpaired, equal variance, two-tailed Student's t tests were used to determine the statistical significance of differences observed between the indicated groups.

Direct Observation of Actin Filament Severing

Unlabeled and Alexa Fluor 488-labeled rabbit muscle actin (Molecular Probes, Eugene, OR) were reconstituted in G-buffer, incubated on ice to depolymerize oligomers, and centrifuged at 100,000 × g to remove aggregates. Protein concentrations were determined using the Coomassie Plus (Bradford) protein assay (Pierce, Rockford, IL). Unlabeled (1.4

μM) and AlexaFluor-488 rabbit actin (0.6 μM) were copolymerized at room temperature for 2 h in ISAP buffer (50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, and 20 mM HEPES-KOH, pH 7.2). Flow chambers were assembled by mounting a 22 mm square coverslip (number 1, Corning, Corning, NY) on a 22 X 40 mm coverslip (number 0, Fisher Scientific, Pittsburgh, PA) with two pieces of Scotch double-sided tape and solutions were loaded via capillary action. The following procedure was adapted from a previous study (Kuhn and Pollard, 2005). Chambers were coated with 10 μg/ml NEM-inactivated myosin (Cytoskeleton, Denver, CO, inactivated as described before (Amann and Pollard, 2001)), for 5 min, and subsequently washed with 10 mg/ml BSA in high salt Tris-buffered saline (50 mM Tris-Cl, pH 7.6, 600 mM NaCl), followed by 10 mg/ml BSA in low salt Tris-buffered saline (50 mM Tris-HCl, pH 7.6, 50 mM NaCl). Polymerized actin was diluted 10-fold and mixed 1:1 in 2X TIR buffer (100 mM KCl, 0.2 mM $MgCl_2$, 2 mM EGTA, 20 mM imidazole, pH 7.0, 100 mM DTT, 0.4 mM ATP, 30 mM glucose, 2% methylcellulose, 40 μg/ml catalase, 200 μg/ml glucose oxidase) just before addition to the flow chamber. The chamber was mounted on the stage of an Olympus 1X-81 inverted microscope (Olympus America, Center Valley, PA) and filaments allowed to settle for 5 min before washing twice with 1X TIR buffer. TgADF and *S. pombe* cofilin were diluted in 1X TIR buffer to 0.3 - 1.5 μM and loaded into the chamber. Filaments were observed by total internal reflection fluorescence (TIRF) microscopy using a 60X Plan Apo objective (oil, numerical aperture of 1.4). Images were captured on a C9100-12 CCD video camera (Hamamatsu Photonics, Bridgewater, NJ) operated and collected by Slidebook software (Intelligent Imaging Innovations, Denver, CO). Frames of 100 ms duration were collected every 5 s and images were processed

and filament lengths measured manually using ImageJ software (http://rsb.info.nih.gov/ij/).

Polymerization Kinetics

Actin (5 μM) was pre-incubated with 0-10 μM TgADF for 10 min before converting Ca-ATP-actin to Mg-ATP-actin with the addition of 10X ME (500 μM $MgCl_2$, 2 mM EGTA) for 5 min. Polymerization was induced with 10X KMEI (500 mM KCl, 10 mM $MgCl_2$, 10 mM EGTA, 100 mM Imidazole HCl, pH 7.0), and light scattering was measured over time in a PTI Quantmaster spectrofluorometer (Photon Technology International, Santa Clara, CA). For experiments with *Toxoplasma* actin, 5 μM phalloidin was also added at the time polymerization was induced.

Steady-state Actin Polymerization

Rabbit actin (2.5-15 μM) was polymerized in the presence of 2.5 molar excess TgADF for 15 h at 25 ^{o}C. Steady-state levels of polymerization were measured by light scattering as above. For *Toxoplasma* actin, polymerization was carried out in the presence of equimolar phalloidin to actin, and 2.5 molar excess TgADF. Light scattering was measured after 9 h at 25 ^{o}C.

Nucleotide exchange

The effect of TgADF on the rate of nucleotide exchange by monomeric actin was assayed using rabbit or Toxoplasma actin labeled with 1,N^6-ethenoadenosine 5'-triphosphate (ε-ATP; Molecular Probes, Eugene, OR) as described (Hawkins *et al.*, 1993). Briefly, G-actin was treated with 20% volume of a 50% slurry of Dowex 1X8 Cl (200 - 400 mesh)

(Sigma Aldrich, St. Louis, MO) to remove excess nucleotide. Actin was subsequently labeled with 200 or 500 μM ε-ATP for 1 h at 4 ^{o}C. Following labeling, unbound ε-ATP was removed with Dowex treatment and 20 μM ε-ATP was added back to prevent actin denaturation. To assay for the effect of TgADF on the rate of nucleotide exchange on Mg-ATP-G-actin, 1 μM actin was pre-incubated with 0 - 20 μM TgADF for 5 min, and the actin was converted to Mg-ATP-actin with the addition of 100 μM $MgCl_2$ and 200 μM EGTA for 5 min. Unlabeled ATP (1.25 mM) was then added to displace ε-ATP from actin and the corresponding loss in fluorescence was monitored over time using a PTI Quantmaster spectrofluorometer (Photon Technology International), excitation = 360 nm, emission = 410 nm. To estimate the apparent affinity of TgADF for monomeric actin, curve-fitting was conducted on the combined data from three experiments, using values that were consistently observed in two or more experiments. The data were fit using nonlinear regression analysis based on first-order exponential decay kinetics using GraphPad Prism (GraphPad software, San Diego, CA).

RESULTS

Comparison of apicomplexan ADFs with other ADF/Cofilin family members

To determine the similarity of apicomplexan ADFs with other ADF/Cofilin (AC) family members, a structure-based multiple sequence alignment was generated (Fig. 1A) using the homology model of the structure of TgADF, based on the known crystal structure of *Acanthamoeba castellani* actophorin (Fig. 1*B*). The apicomplexan ADF proteins are the smallest in the AC family, with TgADF encoding a protein of ~118 amino acids that shares ~63% homology and 39% identity with actophorin, and ~47% homology and ~30% identity with AC isoforms from the higher eukaryotes depicted in Fig. 1A. The predicted structure of TgADF maps onto the structure of actophorin quite well, with the conserved actin binding sites overlapping between the two structures. However a few striking differences were apparent and these are discussed in light of biochemical activities described below.

Actin binding sites identified by site-directed mutagenesis (Lappalainen *et al.*, 1997) and synchrotron protein footprinting (Guan *et al.*, 2002) have previously been mapped on yeast cofilin, and are conserved across ADF/Cofilin family members (Fig. 1*A*, denoted by *asterisk* or *circle*, respectively). These sites are found primarily at the N-terminus, the long α3 helix, and at the C-terminus (Fig. 1*A*). Two regions are identified as being required for interacting with F-actin exclusively (Fig. 1*A*, *black boxes*). Charged residues at the C-terminus in the α4 helix and/or in the C-tail extension are thought to pack against the side of the filament and stabilize the interaction between ADF and the filament. Additionally, the F-loop, consisting of strands β4 and β5, which typically projects out of the AC structure (Fig. 1*B*, *arrow*), is predicted to intercalate within the

filament (Pope *et al.*, 2000). These F-actin binding sites cluster together to form an interface on the opposite side of the molecule from the G-actin binding interface, which is comprised of the α3 helix and the N-terminus (Hatanaka *et al.*, 1996; Fedorov *et al.*, 1997; Leonard *et al.*, 1997).

Apicomplexan ADFs have conserved most of the G-actin binding sites (Fig. 1*A*). However, the sites required exclusively for binding to F-actin are notably absent (Fig. 1*A*, *black boxes*). This is true across the apicomplexan ADFs, with the exception of PfADF2 (Fig. 1*A* and data not shown). Apicomplexan ADFs possess a truncated C-terminus, which may not fold into the terminal α4 helix, and they lack the C-terminal charged residues predicted as necessary for F-actin interactions (Fig. 1*B*). The conserved basic F-loop residue (R78 in *S. pombe* cofilin, G66 in TgADF) shown to be critical for F-actin binding in human and yeast cofilin (Lappalainen *et al.*, 1997; Pope *et al.*, 2000), is also absent in apicomplexan ADFs (Fig. 1*A*, *black boxes*). The F-loop in TgADF and other apicomplexan ADFs (Xu *et al.*, 2008), is itself much shorter compared to other AC proteins due to shorter β4 and β5 strands and a smaller transition between them, and therefore does not project out of the structure (Fig. 1*B arrow*), and presumably would not interact with the filament. These striking differences in the predicted actin binding sites are likely to have important consequences for the activity and function of apicomplexan ADF proteins.

Characterization of *T. gondii* ADF activity

To characterize the activity of *T. gondii* actin depolymerizing factor (TgADF), recombinant protein was expressed and purified in *Escherichia coli*, and tested for its

ability to disassemble actin filaments using actin sedimentation assays (Fig. 2*A*). Since TgADF lacks many key residues required for F-actin binding, the activity of the wild type protein was first established. The effect of TgADF on rabbit actin filaments was analyzed by quantifying the proportion of F-actin sedimenting at 100,000 × *g* after the addition of F-buffer and 1 h incubation with different concentrations of protein. TgADF caused the net disassembly of rabbit actin filaments in a dose-dependent manner, resulting in almost complete disassembly of filaments when present at 2:1 molar excess (Fig. 2*A*). Interestingly, very little TgADF (~8%), co-sedimented with actin filaments at any dose, and this proportion did not increase when higher concentrations of TgADF were used. This is in contrast to what is typically seen with AC proteins such as plant ADF and human cofilin (Carlier *et al.*, 1997; Pope *et al.*, 2000). These data indicated that the disassembly of filaments does not require the stable association of AC proteins with F-actin.

Most AC proteins show pH dependent activity, binding to filaments at neutral pH, and causing the net disassembly of filaments under slightly alkaline conditions (Yonezawa *et al.*, 1985; Hawkins *et al.*, 1993; Hayden *et al.*, 1993). pH regulation has been posited as a mechanism to regulate the activity of AC proteins *in vivo* (Bernstein *et al.*, 2000). Since our polymerization buffer is buffered at the pH permissive for activity, we tested the effect of lower pH on TgADF activity and co-sedimentation with actin filaments. TgADF was equally active at both pH 6.8 and pH 8.2 (Fig. 2*B* & 2*C*). Although more TgADF co-sedimented with filaments at pH 6.8 (~20% compared to ~8% at pH 8.2), this did not affect its filament disassembly activity, perhaps because the fraction co-sedimenting with filaments was still relatively low compared to what is

observed with other AC proteins. Additionally, even though the absolute amount of TgADF co-sedimenting with filaments increased as more protein was added to the reaction, the proportion of TgADF that co-sedimented remained approximately the same or decreased, and this was true at both pHs, suggesting that filament binding was not saturated. Thus, despite lacking some conserved actin binding sites, TgADF was active and able to cause extensive net disassembly of actin filaments, while not stably associating with filaments. Activity was pH independent suggesting that TgADF does not require pH activation.

Comparison of TgADF activity with other ADF/Cofilin proteins, and mutational analysis of actin binding sites

Although TgADF demonstrated potent net filament disassembly activity, we wanted to directly compare its activity to commonly studied AC proteins. *S. pombe* cofilin and *A. castellani* actophorin were chosen as representative proteins to provide a spectrum of activity. *S. pombe* cofilin efficiently disassembles actin filaments by severing, whereas the severing activity of actophorin is relatively weak (Pollard *et al.*, 2000). Both AC proteins were expressed and purified in the same way as TgADF and tested in parallel actin co-sedimentation assays (Fig. 3*A*). All three AC proteins showed negligible sedimentation (1-5% of the total protein) in the absence of actin (data not shown). As seen above, TgADF caused the efficient net disassembly of rabbit actin filaments, with very little protein co-sedimenting with filaments (Fig. 3*A*). In contrast, both *S. pombe* cofilin and actophorin showed very modest net filament disassembly, with *S. pombe* cofilin showing slightly better activity. A maximal effect of 20% less F-actin was

observed with two-fold molar excess *S. pombe* cofilin, compared to 65% less F-actin with two-fold excess TgADF (Fig. 3*A*). Interestingly, approximately 45% of both actophorin and *S. pombe* cofilin co-sedimented with actin filaments (data not shown).

To determine if differences in TgADF activity could be attributed to the lack of F-actin binding sites in TgADF, mutation analysis was used to determine the relative importance of previously defined F-actin binding sites (Fig. 3*B* & 3*C*). Earlier work by Pope *et al.,* (2000), demonstrated that mutating the first (R96 in *S. pombe* cofilin) of the two basic residues in the F-loop region that are critical for F-actin binding, results in human cofilin losing its ability to bind F-actin, and instead causes it to effect the extensive depolymerization of filaments, similar to what was seen with TgADF (Fig. 3*A*). To determine if the lack of a basic residue at this site might play a role in the differential activity of TgADF compared to *S. pombe* cofilin and actophorin, the corresponding point mutations were made in TgADF (G66R and G66K). Conversion to a basic residue at this site had little effect on TgADF co-sedimentation or disassembly of actin filaments (Fig. 3*B*), indicating that this site alone is not sufficient to confer F-actin binding to TgADF.

A second site for F-actin binding is located at the C-terminus of AC proteins, which is truncated in apicomplexan ADFs (Lappalainen *et al.*, 1997). To determine if a more stable interaction between TgADF and F-actin could be reconstituted, the last seven residues of *S. pombe* cofilin were added on to TgADF to generate TgADF-t, and the activity of this protein assessed in the actin sedimentation assay (Fig. 3*B*). Addition of the cofilin tail residues to TgADF had a partial effect on activity, resulting in a two-fold increase in the amount of TgADF-t co-sedimenting with F-actin (data not shown), and decreased disassembly of F-actin (Fig. 3*B*). Addition of the F-loop mutations, G66K or

G66R, to TgADF-t, did not however have any additional effect on the activity or co-sedimentation of the TgADF-t protein with filaments (data not shown). These data indicate that addition of charged residues to the C-terminus of TgADF was able to partially restore binding to F-actin, and this resulted in decreased net filament turnover.

The N-terminus of AC proteins is highly conserved and has previously been identified by mutagenesis in yeast cofilin to be an important site for binding to both F- and G-actin (Lappalainen *et al.*, 1997). In particular the serine 3 residue is an important contact site for interactions with actin, and the activity of some AC proteins is negatively regulated by phosphorylation at this site (Agnew *et al.*, 1995; Moriyama *et al.*, 1996). Although apicomplexan ADFs lack key F-actin binding sites, the N-terminus is very highly conserved with the AC family. To test if this site is important for TgADF activity, point mutations were made at the serine 3 residue. The S3E mutation was made to mimic potential phosphorylation at this site and has been shown to inhibit actin binding for other AC proteins (Agnew *et al.*, 1995; Moriyama *et al.*, 1996), while the S3A mutation was made as a control to show that any loss of activity was due to the introduction of negative charge at this site. These proteins were then compared using the actin sedimentation assay (Fig. 3*C*). Although the S3E mutation resulted in a complete loss of activity, as has previously been seen with the analogous point mutations in human cofilin (Pope *et al.*, 2000), chick ADF (Agnew *et al.*, 1995) and plant ADF (Ressad *et al.*, 1998; Smertenko *et al.*, 1998), the S3A mutation also resulted in a significant loss of TgADF activity, rendering it only 25% as active as the wildtype protein (Fig. 3C). This is in contrast to plant ADF (Smertenko *et al.*, 1998) and actophorin (Blanchoin *et al.*, 2000) where the analogous mutants demonstrate identical or 75% activity compared to the respective

wildtype proteins, indicating that TgADF was particularly sensitive to mutation at this site (Fig. 3*C*). As the amino acid cysteine more closely mimics serine (both small, polar amino acids), serine 3 was mutated to cysteine. This mutant showed wild type activity (Fig. 3*C*) suggesting that the polar nature of this residue was particularly important for actin interactions. Thus, despite differences in the strength of its interaction with F-actin, TgADF appears to still share the same general actin binding sites and these are critical for its activity.

Severing activity of TgADF

S. pombe cofilin is reported to have extremely efficient severing activity (Andrianantoandro and Pollard, 2006). To examine the severing activity of TgADF, TIRF microscopy was used to directly observe filament severing. Rabbit muscle actin was co-polymerized with AlexaFluor-488 actin and filaments were tethered to glass flow-chambers with NEM-inactivated myosin. TgADF or *S. pombe* cofilin were flowed into the chamber at time zero, and time-lapse TIRF microscopy was used to capture severing of filaments over time (Fig. 4).

At a concentration of 0.3 μM, *S. pombe* cofilin caused the rapid disassembly of actin filaments within the first 3 min (Fig. 4*A*, SpCOF, *top*). After 6 min there was little further change, with severed fragments drifting out of view, occasionally leaving behind longer stable fragments that remained attached to the surface of the chamber (Fig. 4*A*, SpCOF, *top*). In contrast, very little activity was observed with 0.3 μM TgADF (Fig. 4*A*, *middle*), except for the occasional breakage of longer filaments after 9 min. To determine if TgADF had any severing capacity, activity was examined at five-fold higher

concentrations of TgADF. At 1.5 μM TgADF (Fig. 4*A*, *bottom*), severing was clearly visible after 6 min, indicating that TgADF can sever filaments when present at higher concentrations.

To quantitate the relative rate of filament disassembly for both TgADF and SpCOF under these conditions, the average length of the longest class of filaments was calculated over time (Fig. 4*B*). TgADF at a concentration of 1.5 μM was found to disassemble actin filaments at a rate approximately half that of 0.3 μM *S. pombe* cofilin (initial rates of approximately 1.1 ± 0.442 μm/min compared to 2.0 ± 0.404 μm/min respectively). To directly visualize severing activity, single filaments in the presence of 1.5 μM TgADF were tracked over time (Fig. 4*C*). Severing activity was visualized as a break in the filament and the subsequent fragmentation into more pieces (Fig. 4*C*).

Interaction of TgADF with *T. gondii* actin filaments during sedimentation

Studying the interaction of TgADF with a heterologous protein such as rabbit actin is useful to provide a frame of reference for identifying the salient properties of TgADF in comparison to other ADF/Cofilin family members. However, to determine the biologically relevant functions of TgADF it was important to examine its interaction with its homologous actin substrate. *T. gondii* expresses one actin allele, TgACT1, which shares 83% identity with rabbit actin (Dobrowolski *et al.*, 1997). Actin sedimentation assays were used to investigate how TgADF interacts with TgACT filaments.

As previously reported, *T. gondii* actin is inherently unstable, and unlike higher eukaryotic actins, the addition of F-buffer does not induce formation of long stable filaments that sediment at 100,000 × *g* (Fig. 5*A*) (Sahoo *et al.*, 2006). However, stable

TgACT filaments can be rescued when TgACT is polymerized in the presence of equimolar phalloidin (Fig. 5*A*)[2]. Small actin oligomers that formed in F-buffer were also sedimented by centrifugation at 350,000 × *g*, as described previously for *Toxoplasma* and *Plasmodium* actins (Schmitz *et al.*, 2005; Sahoo *et al.*, 2006). The interaction between TgADF and TgACT filaments was examined under these three conditions.

In the absence of phalloidin, only 20-30% of the TgACT in F-buffer sedimented at 100,000 × *g*, and TgADF had little effect on this behavior (Fig. 5*B*). In the presence of equimolar phalloidin in F-buffer, there was a 50% increase in the amount of TgACT sedimenting at 100,000 × *g*, and a two-fold molar excess of TgADF had only a modest effect on this population (Fig. 5*B*). However, when TgACT was polymerized by the addition of F-buffer and centrifuged at 350,000 × *g* to pellet small oligomers, 70-80% of the total actin was pelleted (Fig. 5*A*). A two-fold molar excess of TgADF was able to disassemble 50% of the actin that pelleted under these conditions (Fig. 5*B*). Interestingly, TgADF did not co-sediment with TgACT under any of the conditions tested indicating that it does not stably associate with TgACT filaments or oligomers (Fig. 5*A*). This is in contrast to *S. pombe* cofilin which co-sedimented with TgACT at 350,000 × *g* (data not shown). These data indicate that TgADF can efficiently disassemble small TgACT oligomers.

Effects of TgADF on actin polymerization kinetics

The data thus far suggested that TgADF had a weak affinity for actin filaments, yet it could disassemble small oligomers of TgACT1 and rabbit actin filaments. To investigate the effect of TgADF on actin polymerization, light scattering was used. Actin was

incubated with TgADF for 5 min before polymerization was induced with the addition of KMEI-buffer. The increase in light scattering was measured over time (Fig. 6*A*). At ≤ 1:1 molar ratio TgADF:rabbit actin, there was an increase in the initial rate of polymerization with TgADF (Fig. 6*A*). This could be due to weak severing of filaments, which would generate more seeds for elongation. Alternatively, transient binding of TgADF to filaments could cause an increase in light scattering. However, when present in two-fold molar excess, TgADF inhibited the nucleation and polymerization of rabbit actin filaments, suggesting that TgADF was sequestering actin monomers. When TgACT was used as the substrate and polymerization induced by the addition of KMEI buffer and equimolar phalloidin, TgADF inhibited polymerization at all doses, even when extremely low TgADF concentrations were used (Fig. 6*B*). The more potent effect of TgADF on TgACT polymerization compared to rabbit actin could be due to higher affinity interactions between TgADF and TgACT, or because TgACT polymerization was slower and more sensitive to inhibition. These data demonstrated that TgADF strongly inhibited TgACT polymerization even at low doses, and this occurred much more efficiently than with rabbit actin.

To test if TgADF was able to sequester G-actin, the effect of TgADF on steady-state actin polymerization was examined (Fig. 7). Rabbit actin was polymerized in the presence of 2.5 molar excess TgADF or *S. pombe* cofilin, and steady-state polymerization was measured by light scattering (Fig. 7*A*). TgADF inhibited the steady-state polymerization of at least 15 μM rabbit actin. In contrast, *S. pombe* cofilin caused an increase in light scattering at actin concentrations of 10 μM and above. This increased light scattering could be due to enhanced polymerization in the presence of *S. pombe*

cofilin, which has been shown to nucleate filaments when present at two-fold molar excess , and/or from *S. pombe* cofilin binding to filaments and leading to increased mass. When the effect of TgADF on steady-state TgACT polymerization was examined (Fig. 7*B*), TgADF reduced the extent of steady-state polymerization at all actin concentrations tested. This clearly indicates that TgADF inhibits actin polymerization by sequestering actin monomers.

Effects of TgADF on nucleotide exchange of G-actin

To demonstrate a direct interaction between TgADF and G-actin, we measured the effect of TgADF on the exchange of nucleotide bound to actin monomers (Fig. 8). AC proteins typically bind to G-actin and inhibit the exchange of actin bound nucleotides (Nishida, 1985; Hawkins *et al.*, 1993; Hayden *et al.*, 1993; Yamashiro *et al.*, 2005; Andrianantoandro and Pollard, 2006), with the exception of *Plasmodium* ADF1 and *Tetrahymena* ADF73p which were recently shown to stimulate nucleotide exchange (Schuler *et al.*, 2005b; Shiozaki *et al.*, 2009). Monomeric actin was labeled with the ATP analogue ε-ATP, which fluoresces when bound to actin, and the rate of nucleotide exchange was measured as a decrease in fluorescence when the actin-bound ε-ATP was displaced with unlabeled ATP. In the absence of TgADF, the nucleotide exchange rate of TgACT was ~2-3-fold faster than RbACT (Fig. 8*A* & 8*B*). However, in the presence of TgADF, nucleotide exchange was inhibited on both RbACT and TgACT monomers in a dose-dependent manner (Fig. 8*A* & 8*B*). Interestingly, at low ratios (<1:1) of TgADF and TgACT, an approximately two-fold increase in the initial rate of nucleotide exchange was often observed (Fig. 8*B*, *green curve* (0.25 μM TgADF) compared to *dark blue curve* (0

μM)), suggesting that at very low concentrations, TgADF may stimulate nucleotide exchange. Using the dose-dependent inhibition of nucleotide exchange in the presence of TgADF, we estimated the apparent affinity of TgADF for TgACT and RbACT monomers to be approximately 0.81 and 0.64 μM, respectively. These data demonstrate a direct interaction of moderate affinity between TgADF and ATP-actin monomers, providing additional support for the sequestering activity of TgADF.

DISCUSSION

To determine the mechanism by which TgADF accelerates actin filament turnover, we analyzed its biochemical interactions with both mammalian and *T. gondii* actin *in vitro*. Although severing is typically the main mechanism by which AC proteins are thought to effect filament turnover (Maciver, 1998), TgADF was a comparatively weak severing protein. We found that the absence of key F-actin binding sites in TgADF was associated with high net filament disassembly activity. We demonstrate that the primary mechanism for the efficient net disassembly of actin filaments by TgADF is due to the strong sequestering of actin monomers. These properties identify an ADF that is adapted to function in a primarily G-actin rich environment, where filaments are rare and rapidly assemble and turn over for very specific biological processes.

Two sites have previously been identified on AC proteins as critical for F-actin interactions: charged residues at the C-terminus of the protein (Lappalainen *et al.*, 1997; Ono *et al.*, 2001), and two basic residues in the F-loop (Lappalainen *et al.*, 1997; Pope *et al.*, 2000) that extends out of the crystal structure of AC proteins. Most apicomplexan ADFs, excluding *Plasmodium* PfADF2, are deficient at both sites. Apicomplexan ADFs are truncated at the C-terminus and therefore lack the C-terminal α4 helix and the C-tail extension. The first of the two basic residues in the F-loop is also missing in all apicomplexan ADFs. In addition to this, the apicomplexan ADFs that have been examined by homology modeling (present study and (Xu *et al.*, 2008)), appear to contain a very short F-loop, that likely does not promote tight binding to the filament. Consistent with the lack of key F-actin binding sites in its molecular structure, TgADF displayed

limited co-sedimentation with actin filaments, indicating the absence of stable interactions with filaments. This property is unusual for proteins in the AC family (Carlier *et al.*, 1997; Maciver *et al.*, 1998; Pope *et al.*, 2000). However, the lack of a stabilizing interaction with F-actin was associated with a greater effect on the net disassembly of actin filaments. Addition of the C-terminal F-actin binding site from *S. pombe* cofilin to TgADF stabilized TgADF's interaction with F-actin, but concomitantly decreased the net filament disassembly activity, indicating that the two properties are inversely related. Restoration of the conserved basic F-loop residue to TgADF had no effect on its activity. However, this is likely due to the F-loop being considerably shorter in TgADF, which may not facilitate contact with the filament even when these positive charges are restored. Such high net filament disassembly activity has only been previously observed with a very small subset of AC proteins (Unc60A (Yamashiro *et al.*, 2005), human ADF (Hawkins *et al.*, 1993), chick ADF (Hayden *et al.*, 1993), and echinoderm depactin (Mabuchi, 1983)). This activity is typically pH-dependent, with human and chick ADF binding to the filaments between pH 6.8 –7.1, and causing net disassembly above pH 7.5 (Hawkins *et al.*, 1993; Hayden *et al.*, 1993) (pH-dependency has not been examined for depactin). In contrast, TgADF displayed potent activity at both the permissive and non-permissive pH, suggesting that wild type TgADF did not have the features to strongly interact with actin filaments.

S. pombe cofilin and actophorin are two well-characterized members of the AC family, and group into the cofilin-like class of AC proteins as described by (Chen *et al.*, 2004). Both proteins co-sedimented with actin filaments and showed only modest net filament disassembly, even at the pH permissive for activity. AC proteins that can bind

better to F-actin may be more effective at filament severing, and there is some data to support this model (Ono *et al.*, 2001). When the severing activity of TgADF was compared with *S. pombe* cofilin, TgADF was found to be a comparatively weak severing protein, with five-fold higher concentrations of TgADF yielding a filament disassembly rate that was still two-fold less than *S. pombe* cofilin. Our data suggest that TgADF requires much higher concentrations to sever filaments than is typical for AC proteins (Andrianantoandro and Pollard, 2006). The severing activity of TgADF was similar to the reported activity of the worm AC homologue Unc60A, which also requires concentrations as high as 2 μM to detect severing (Yamashiro *et al.*, 2005). The requirement for comparatively high concentrations of TgADF to detect severing, and the absence of stable association with actin filaments, suggested that TgADF had a low affinity for actin filaments, and that severing alone could not be the primary mechanism by which TgADF caused the efficient net disassembly of actin filaments.

The activities of most other apicomplexan actin binding proteins have been suggested based solely on their interactions with heterologous actin proteins (Baum *et al.*, 2008; Plattner *et al.*, 2008; Ganter *et al.*, 2009). Since key differences exist between the molecular structure of apicomplexan actins compared to conventional eukaryotic actins (Sahoo *et al.*, 2006), we wanted to confirm that TgADF interactions relevant to the apicomplexan actin system were being captured. Using recombinant *T. gondii* actin (TgACT) we found that TgADF efficiently disassembled short TgACT oligomers that sedimented at 350,000 × *g*. Interestingly, TgADF failed to co-sediment with TgACT at either 100,000 × *g* or 350,000 × *g*, indicating that the failure to co-sediment with rabbit actin filaments was not due to potential structural differences between the heterologous

and homologous substrates, but rather that TgADF does not interact strongly with F-actin. The disassembly of short TgACT oligomers was a specific activity of TgADF, as *S. pombe* cofilin did not cause the disassembly of these oligomers, and instead co-sedimented, and increased the proportion of TgACT that sedimented at 350,000 × *g* (data not shown). The limited interaction of TgADF with actin filaments and oligomers, while causing their efficient disassembly, suggested that TgADF had a weak affinity for polymerized actin, but a strong affinity for actin monomers.

To directly ascertain the result of TgADF interactions with actin monomers, we monitored its effect on actin polymerization. Low concentrations of TgADF efficiently inhibited the polymerization of TgACT, with an almost complete inhibition of polymerization in the presence of equimolar TgADF. A qualitatively similar result was seen with rabbit actin, although excess TgADF was required to observe this effect. This inhibition of polymerization is in contrast to most AC proteins, which typically cause increased polymerization or overshoot kinetics with increasing amounts of protein (Carlier *et al.*, 1997; Ono and Benian, 1998). However, an inhibitory effect on actin polymerization has previously been observed with the *C. elegans* AC homologue Unc60A (Ono and Benian, 1998), embryonic chicken skeletal muscle ADF (Abe and Obinata, 1989), and echinoderm depactin (Mabuchi, 1983). Similar to the effect of TgADF on TgACT polymerization, Unc60A and embryonic chick ADF inhibit actin polymerization at substoichiometric concentrations in a dose-dependent manner (Abe and Obinata, 1989; Yamashiro *et al.*, 2005). Additionally, when present at a 1:1 or 2:1 molar ratio with actin, Unc60A strongly inhibits actin nucleation, similar to what is seen with TgADF (Yamashiro *et al.*, 2005). In contrast, echinoderm depactin causes an initial

overshoot in actin polymerization before inhibiting overall polymerization levels, and this overshoot is possibly due to severing activity, making more free ends available for polymerization (Mabuchi, 1983).

As the delay in the nucleation phase of polymerization was suggestive of monomer sequestration, steady-state polymerization assays were done in the presence of TgADF to further investigate this. The steady-state polymerization of up to 15 μM actin (for both *T. gondii* and rabbit actin) was inhibited in the presence of TgADF. This assay conclusively demonstrated that TgADF primarily interacts with actin by sequestering actin monomers. This fairly unusual finding of strong sequestering activity by an AC protein has previously only been directly shown for Unc60A (Yamashiro *et al.*, 2005), which also inhibits the steady-state polymerization of at least 15 μM actin, and for the embryonic chicken skeletal muscle ADF (Abe and Obinata, 1989), which inhibits the steady-state polymerization of actin at substoichiometric concentrations (as determined by reduced viscosity measurements, and higher G-actin concentrations).

To demonstrate a direct interaction between TgADF and monomeric actin, we examined the effect of TgADF on the rate of nucleotide exchange by G-actin. AC proteins typically bind to and inhibit nucleotide exchange by G-actin (Nishida, 1985; Hawkins *et al.*, 1993; Hayden *et al.*, 1993), and TgADF was found to similarly inhibit nucleotide exchange by both TgACT and rabbit actin in a dose-dependent manner. Based on this, the apparent affinity of TgADF for Mg-ATP-G-actin was calculated to be 0.81 and 0.64 μM for TgACT and rabbit actin, respectively. Although most AC proteins bind to ADP-G-actin with affinities of 0.5-1 μM, there is a 10-20 fold decrease in the affinity for Mg-ATP-G-actin (Chen *et al.*, 2004). In contrast, the higher affinity of TgADF for

Mg-ATP-G-actin is a property shared with the other monomer sequestering AC proteins. For example, Unc60A and chick ADF have affinities for Mg-ATP-G-actin of approximately 1.6 μM and 1 μM, respectively (Chen *et al.*, 2004; Yamashiro *et al.*, 2005). The higher affinity of these AC proteins is consistent with the observed sequestering of Mg-ATP-G-actin, which is thought to be the predominant form of G-actin *in vivo* (Chen *et al.*, 2004).

Although strong depolymerizing activity was the basis for how many ADF proteins were originally identified (Bamburg *et al.*, 1980; Mabuchi, 1983; Nishida *et al.*, 1985), the mechanism for how depolymerization was occurring was not defined. Although there is no obvious signature in the amino acid sequence that distinguishes the AC proteins with strong monomer sequestering activity as being more similar, structural features that suggest the lack of, or perturbation to F-actin binding sites may be a unifying property. This is observed with TgADF, which lacks known F-actin binding sites, and Unc60A, in which the surface exposed insertion of charged residues (Ono and Benian, 1998) may disrupt filament interactions. Another shared feature of this sub-type of AC isoforms, is their expression in G-actin rich environments (Bamburg *et al.*, 1980; Harris *et al.*, 1980; Mabuchi, 1983; Nishida *et al.*, 1985). In the case of embryonic chicken skeletal muscle ADF, its expression has been shown to correlate with the G-actin content in the cell (Abe and Obinata, 1989). In embryonic cells, a large proportion of the actin is present as G-actin (Shimizu and Obinata, 1986). ADF is abundantly expressed in these cells, and both inhibits actin polymerization and depolymerizes F-actin (Abe and Obinata, 1989). As the skeletal muscle develops, the proportion of F-actin in the cell increases and the amount of actin turnover decreases (Abe and Obinata, 1989). This

change in the actin milieu also corresponds to a drop in ADF expression (Abe and Obinata, 1989), suggesting that the ADF isoform is expressed when rapid changes in the actin cytoskeleton are required.

In *T. gondii*, 98% of the actin is unpolymerized (Dobrowolski *et al.*, 1997; Wetzel *et al.*, 2003; Sahoo *et al.*, 2006), yet the parasite depends on filamentous actin to achieve rapid rates of motility with speeds of ~1-10 μm/s (Håkansson *et al.*, 1999), and to productively invade host cells. This scenario is similar to the actin dynamics in the embryonic skeletal muscle described above, in that most of the actin is in the G-form, and filaments must be rapidly assembled and disassembled. We predict that TgADF plays a key role in regulating this dynamic actin equilibrium in the parasite, through its interaction with both F- and G- actin. The moderate affinity of TgADF for TgACT monomers, and the presence of equimolar concentrations of TgADF and TgACT in the parasite (~8-10 μM, data not shown), suggest that TgADF will have an important role in sequestering actin monomers in the parasite. Additionally, profilin and cyclase-associated protein (CAP), the other highly conserved G-actin binding proteins found in apicomplexan parasites (Baum *et al.*, 2006; Schüler and Matuschewski, 2006), may play important roles in maintaining the parasite actin monomer pool. TgProfilin has previously been shown to be essential for gliding motility and to interact with actin from parasite lysate (Plattner *et al.*, 2008), but the affinity of this interaction is not known. Although it has weak sequestering activity with heterologous actin, the biochemical interaction between TgProfilin and TgACT has not yet been examined (Plattner *et al.*, 2008). Similarly, little is known about the biochemical activities of TgCAP. Further studies will

be necessary to assess the relative roles of all three proteins in buffering the substantial G-actin pool in the parasite.

Parasite actin must be rapidly turned over in order to generate productive gliding motility, as jasplakinolide treated parasites with hyper-stabilized filaments are unable to undergo directional movement (Wetzel *et al.*, 2003). TgADF is an excellent candidate for controlling filament turnover based on its ability to sever actin filaments and promote their net disassembly. The relatively weak severing activity of TgADF may provide a mechanism to regulate this activity, so that TgADF only functions as a severing protein when present at high local concentrations in the parasite. Alternatively, a lower level of activity may be sufficient to efficiently sever less stable TgACT filaments. This situation shares parallels with the worm system, where two ADF isoforms (Unc60A and Unc60B) are expressed in different tissues. The Unc60A isoform shows similar properties to TgADF, with strong sequestering activity and weak severing activity (Yamashiro *et al.*, 2005). Unc60A is expressed in early embryos and is required for embryonic cytokinesis, an environment where actin is likely to be undergoing rapid turnover (Ono *et al.*, 2003). Ono *et al.,* (2008) recently demonstrated that a knock down of Unc60A is unable to be functionally complemented by Unc60B, the differentially spliced variant of Unc60A, which has strong severing activity. However, Unc60B mutants that have weak severing activity are able to rescue Unc60A knock down cells, suggesting that the property of weak severing is functionally important in cells expressing Unc60A (Ono *et al.*, 2008). This suggests that weak severing activity is likely to be an important property when filaments are transient and are being turned over rapidly, as strong severing activity may prevent transient filaments from being stable long enough to carry out their function. We

predict that weak severing will also be an important property of *T. gondii* ADF for controlling actin turnover during gliding motility.

Recent work has demonstrated the essential nature of various actin binding proteins in apicomplexan parasites (Schuler *et al.*, 2005b; Plattner *et al.*, 2008; Ganter *et al.*, 2009), and the importance of careful regulation of the actin machinery for productive gliding motility, a process essential for host cell invasion and successful completion of the parasite life-cycle. In this study, we found that TgADF is potent at sequestering actin monomers *in vitro*, and has weak filament severing activity. The ability to both maintain high concentrations of G-actin and to regulate the turnover of actin filaments, positions ADF to play a critical role in regulating the unique actin dynamics found in apicomplexan parasites. In addition to this, our work points to an underappreciated role for ADF proteins as monomer-sequestering proteins in G-actin rich environments.

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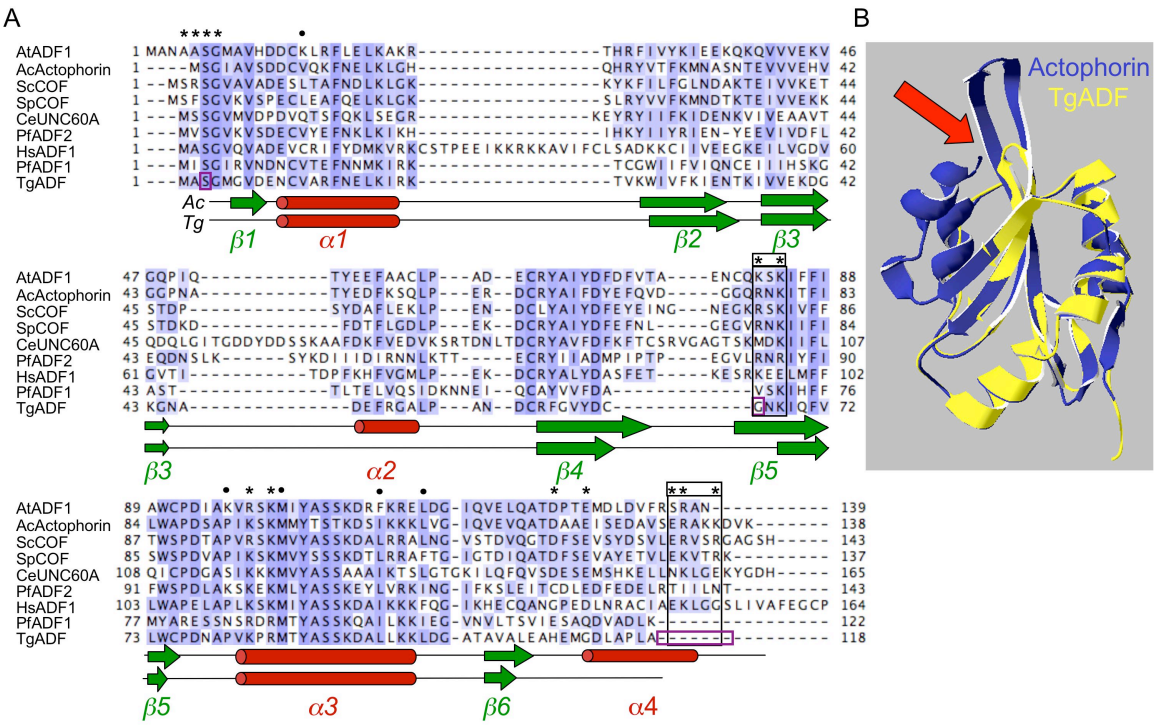
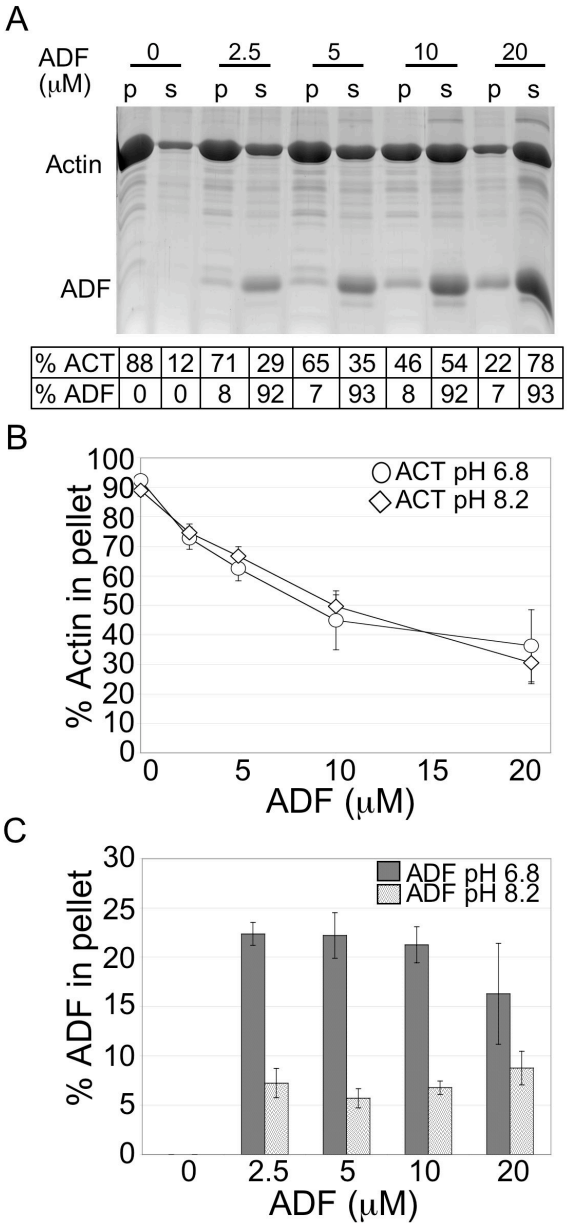


FIGURE 1. Comparison of apicomplexan ADFs with representative ADF/Cofilin proteins. *A,* Clustal X alignment of ADF/Cofilin family members highlighting key features. Actin binding sites identified in *Saccharomyces cerevisiae* cofilin by mutagenesis (29) or synchrotron protein footprinting (34) indicated by *asterisk* or *circle* respectively. Residues required exclusively for F-actin binding are boxed in *black*. Serine 3, Glycine 66 and C-terminal residues that were mutated or added back in TgADF to analyze the actin binding sites in Fig. 3, are boxed in *purple*. Sequences shown: AtADF1: *Arabidopsis thaliana* ADF1, AcActophorin: *Acanthamoeba castellani* actophorin, ScCOF: *Saccharomyces cerevisiae* cofilin, SpCOF: *Schizosaccharomyces pombe* cofilin, CeUnc60A: *Caenorhabditis elegans* Unc60A, PfADF2: *Plasmodium falciparum* ADF2, HsADF: *Homo sapiens* ADF, PfADF1: *Plasmodium falciparum* ADF1, TgADF: *Toxoplasma gondii* ADF. *B,* Homology model of *T. gondii* ADF (TgADF, shown in

yellow) based on *A. castellani* actophorin (Actophorin, PDB entry 1ahq, shown in *blue*). *Arrow* points to short F-loop in TgADF compared to actophorin.



% ACT	88	12	71	29	65	35	46	54	22	78
% ADF	0	0	8	92	7	93	8	92	7	93

FIGURE 2. Characterization of *Toxoplasma* ADF activity. *A,* Dose-dependent disassembly of rabbit actin filaments by TgADF. A representative Sypro-Ruby stained gel showing the effect of increasing concentrations of TgADF on the amount of F-actin pelleting at 100,000 × *g*. Quantitation of the proportion of actin and TgADF in the pellet and supernatant fractions is given below. Rabbit actin (10 μM) was polymerized into filaments by the addition of F-buffer, before incubation with TgADF (0-20 μM). Samples

were centrifuged (100,000 × *g*) to sediment actin filaments, and the pellet (*p*) and supernatant (*s*) fractions analyzed by SDS-PAGE. Bands were quantified by phosphorimager analysis of Sypro-Ruby stained gels. *B,* Effect of pH on the disassembly of filaments by TgADF. Quantitation of the proportion of actin in the pellet fraction after interaction with TgADF at pH 6.8 or pH 8.2. Rabbit actin was polymerized under normal conditions (pH 8) and interacted with TgADF at either pH 6.8 or pH 8.2 (N=3 experiments, mean ± S.E.M.). *C,* Effect of pH on TgADF co-sedimentation with actin filaments. Quantitation of the proportion of TgADF in the pellet fraction after interaction with rabbit actin at pH 6.8 or pH 8.2 (N=3 experiments, mean ± S.E.M.).

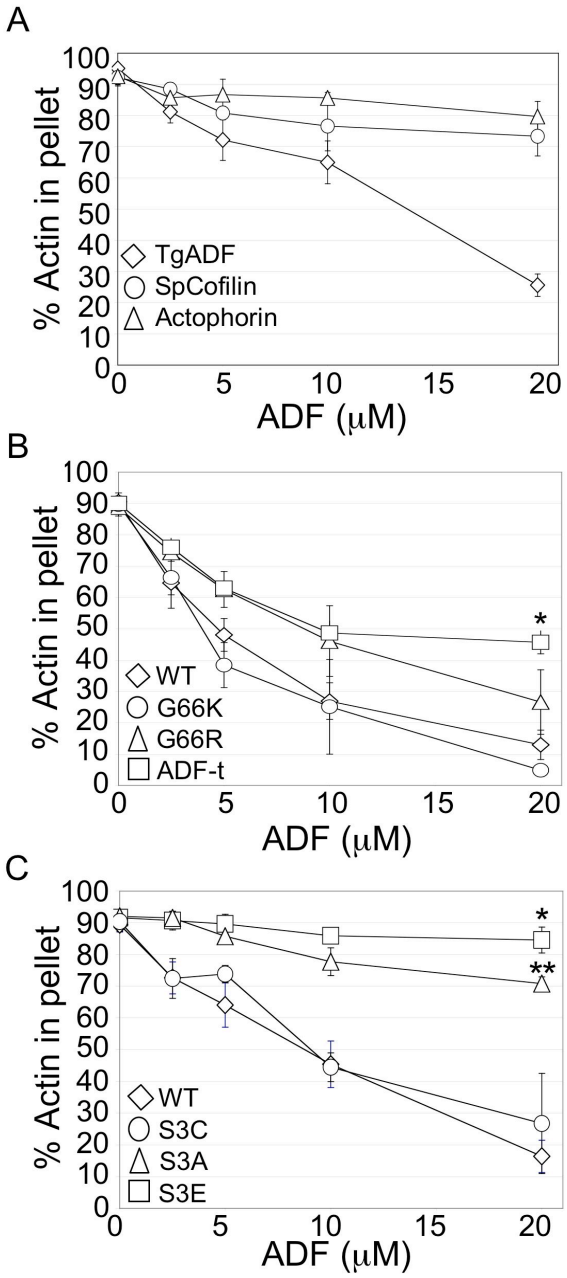


FIGURE 3. Comparison of TgADF activity with other ADF/Cofilin proteins, and mutational analysis of actin binding sites. *A,* Comparison of TgADF activity with ADF/Cofilin proteins *S. pombe* cofilin and *A. castellani* actophorin. Quantitation of the proportion of actin sedimenting at 100,000 × *g* after polymerization by the addition of F-

buffer and incubation with TgADF, *S. pombe* cofilin (SpCofilin) or *A. castellani* actophorin (Actophorin). Experiments were done as described in Fig 2. (N=3 experiments, mean ± S.E.M.). *B,* Effect of putative F-actin binding sites on TgADF activity. The filament disassembly activity of TgADF expressing the conserved basic F-loop residue (G66K or G66R) or the C-terminal residues of *S. pombe* cofilin (ADF-t) was compared to wild-type (WT) TgADF. The graph shows the relative proportion of actin sedimenting at 100,000 × *g* (N=3 experiments, mean ± S.E.M., * P <0.005 Student's *t* test, ADF-t vs. WT). *C,* Activity of TgADF Serine 3 mutants. Actin filament disassembly activity of TgADF with mutations at the serine 3 residue to cysteine (S3C), alanine (S3A) or glutamic acid (S3E), were compared to WT TgADF (WT). The graph shows the relative proportion of actin sedimenting at 100,000 × *g* (N=3 experiments, mean ± S.E.M., * P <0.001 Student's *t* test, S3E vs. WT, ** P <0.05 Student's *t* test, S3A vs. S3E).

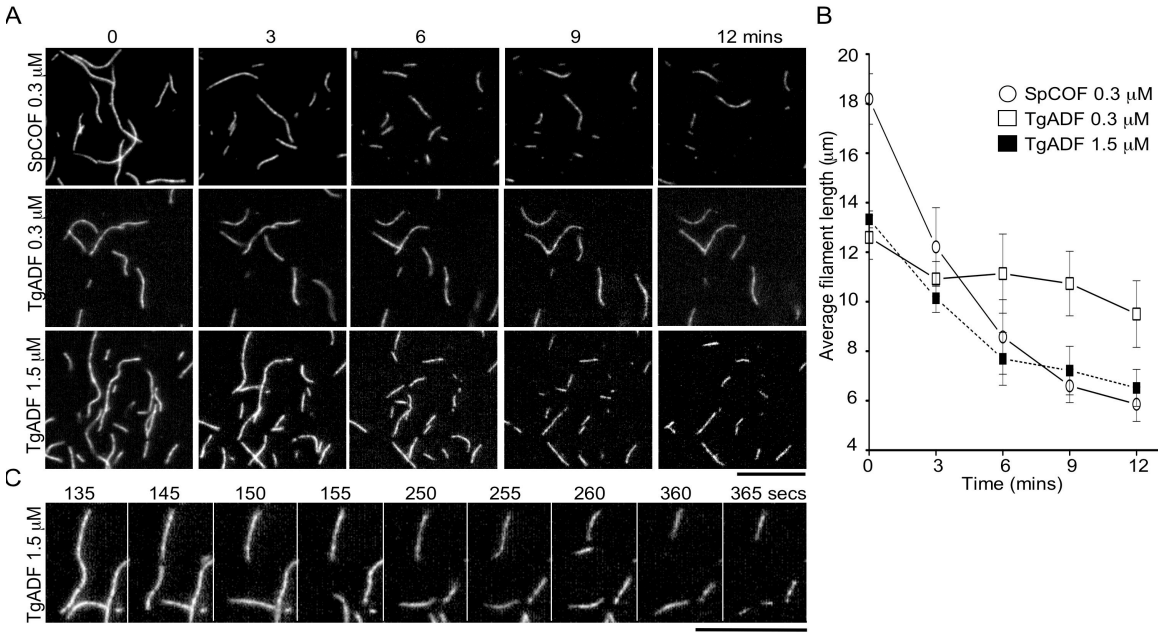


FIGURE 4. Severing activity of TgADF as observed by TIRF microscopy. *A,* Severing of actin filaments by TgADF and *S. pombe* cofilin. Fluorescence time-lapse micrographs of actin filaments taken over a period of 0-12 min after the addition of 0.3 μM *S. pombe* cofilin (SpCOF, *top*), 0.3 μM TgADF (*middle*), or 1.5 μM TgADF (*bottom*) at time zero. Rabbit actin copolymerized with AlexaFluor 488-labeled actin was tethered to flow chambers with NEM-treated myosin. Time-lapse TIRF microscopy was used to visualize filament severing by TgADF and *S. pombe* cofilin over time. *Scale bar* is 10 μm. *B,* Quantitation of the rate of filament disassembly by TgADF and *S. pombe* cofilin. The average length (mean ± S.E.M.) of the 15 longest filaments in the field of view was calculated at the indicated timepoints after TgADF or SpCOF addition, and plotted for each condition (N=3 experiments). *C,* Detailed montage of actin filaments being severed by 1.5 μM TgADF over time. *Scale bar* is 10 μm.

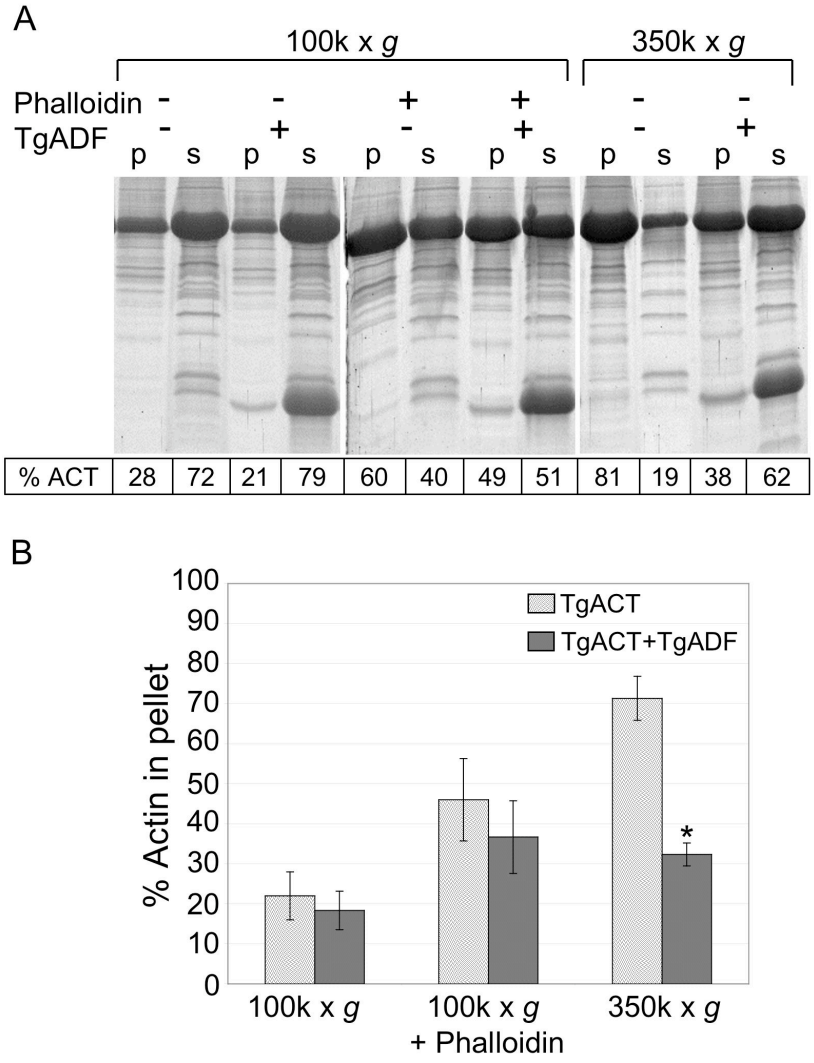


FIGURE 5. Interaction of TgADF with *T. gondii* actin filaments during sedimentation. *A,* Effect of TgADF on the sedimentation activity of *T. gondii* actin filaments polymerized by the addition of F-buffer. A representative Sypro-Ruby stained SDS-PAGE gel showing the proportion of *T. gondii* actin sedimenting at 100,000 × *g* or 350,000 × *g* in the absence and presence of phalloidin or TgADF. Quantitation of the actin bands is indicated in the table below. *P* = pellet, *S* = supernatant. *B,* Quantitation of the percentage of actin in the pellet fraction under the conditions in (*A*) based on 3

independent experiments, mean ± S.E.M., * P <0.005 Student's t test, TgACT alone *vs.* TgACT + TgADF.

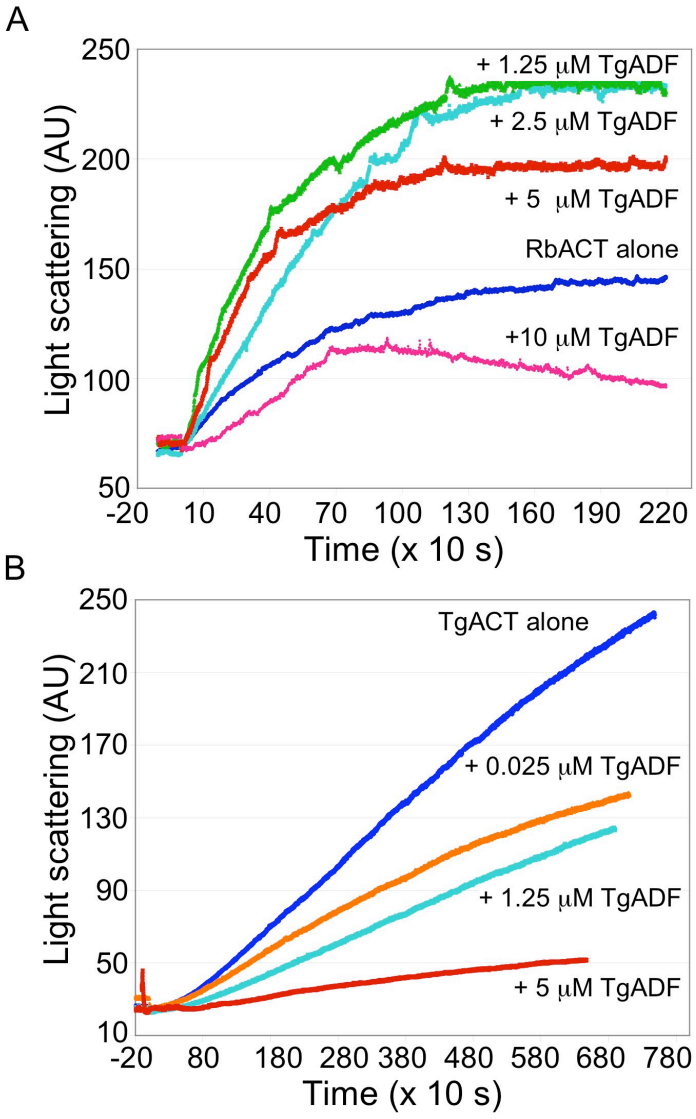


FIGURE 6. Effects of TgADF on actin polymerization kinetics. *A,* Effect of TgADF on rabbit actin polymerization. Polymerization of rabbit actin (RbACT, 5 μM) was measured by light scattering in the presence of 0-10 μM TgADF. Rabbit actin was incubated with TgADF for 10 min prior to initiation of polymerization with the addition of 1/10th the volume of 10X KMEI. A representative experiment is shown (N=3). *B,* Effect of TgADF on *T. gondii* actin polymerization. Polymerization of *T. gondii* actin (TgACT, 5 μM) in the presence of 0–5 μM TgADF was measured over time by light

scattering. Experiments were done as in (*A*), with 5 μM phalloidin added at the time of polymerization, to stabilize *T. gondii* actin filaments. A representative experiment is shown (N=2).

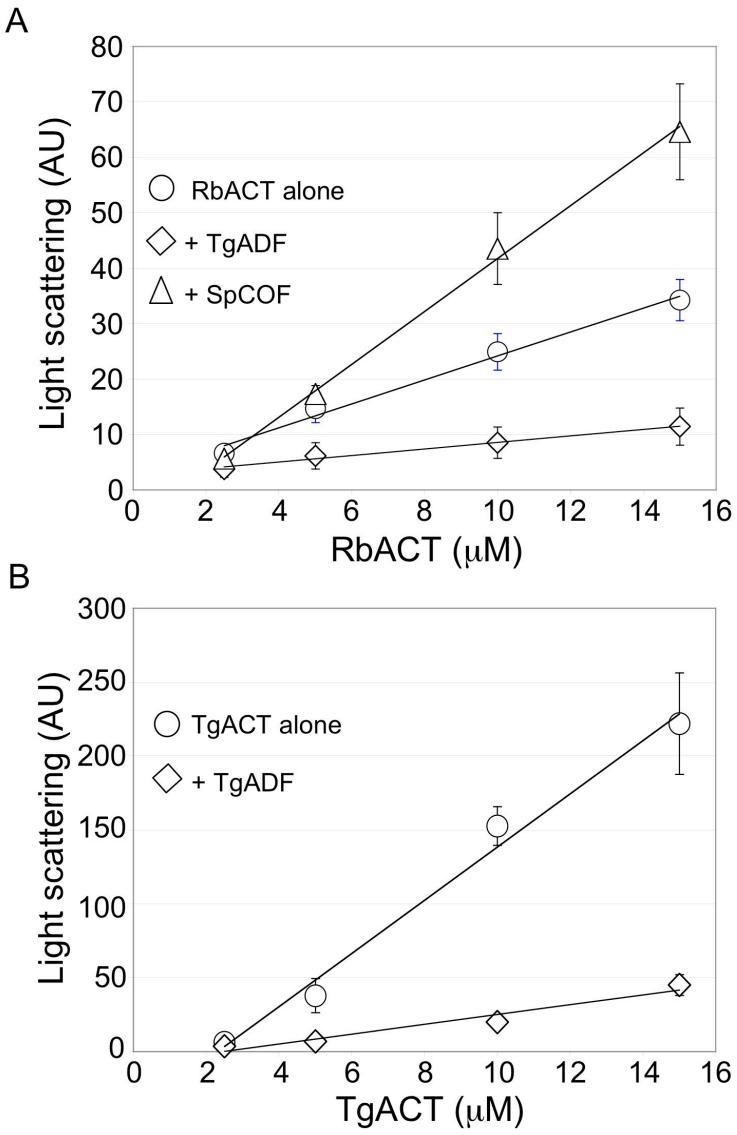


FIGURE 7. Effect of TgADF on steady-state actin polymerization. *A,* Effect of TgADF on the steady-state polymerization of rabbit actin as measured by light scattering. Varying concentrations of rabbit actin (RbACT, 2-15 μM) were polymerized in KMEI buffer, in the presence of 2.5 molar excess TgADF or *S. pombe* cofilin (SpCOF) at 25 ^{o}C until steady-state was achieved. The data represents the average (mean ± S.E.M.) of three independent experiments. *B,* Effect of TgADF on *T. gondii* actin steady-state polymerization as measured by light scattering. Varying concentrations of *T. gondii* actin

(TgACT, 2-15 μM) were polymerized in KMEI buffer, in the presence of equimolar phalloidin and 2.5 molar excess TgADF at 25 ^{o}C until steady-state was achieved. The data represents the average (mean ± S.E.M.) of three independent experiments.

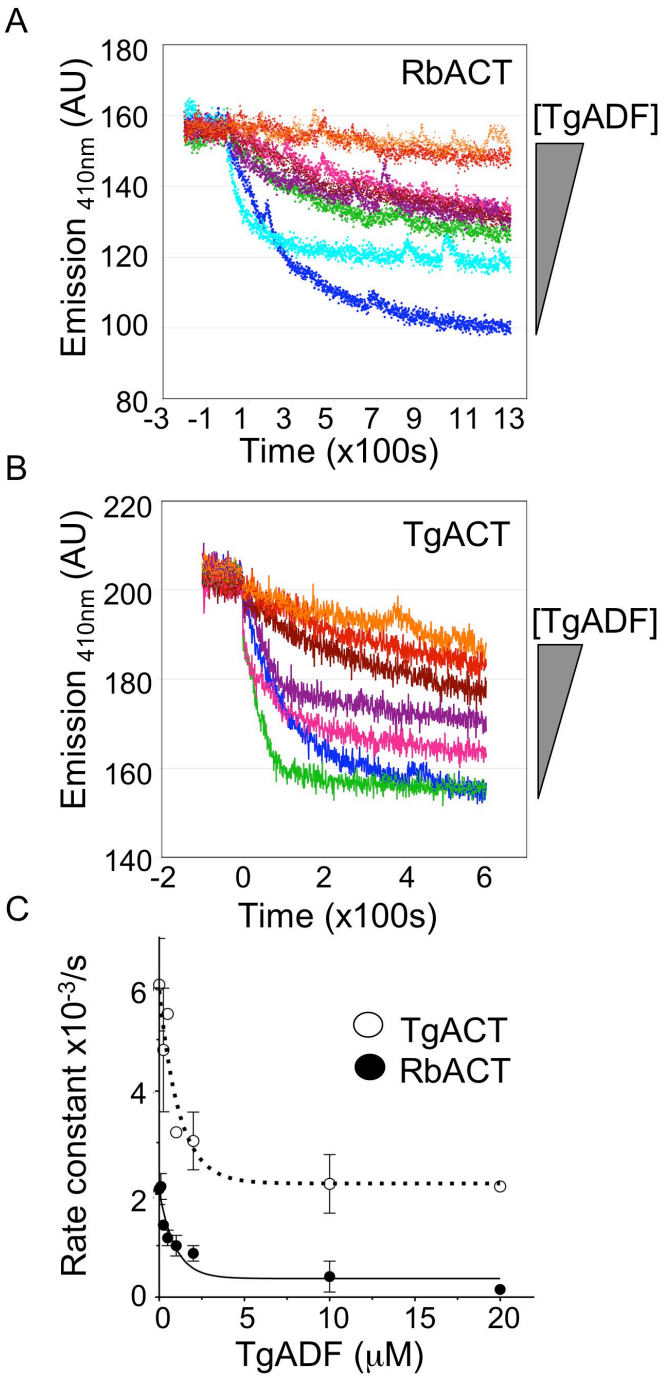


FIGURE 8. Effect of TgADF on nucleotide exchange of G-actin. *A*, Effect of TgADF on the nucleotide exchange rate of monomeric rabbit actin (RbACT). The nucleotide exchange rate of ε-ATP-labeled Mg-actin monomers (1 μM), in the presence of varying concentrations of TgADF (0-20 μM), was monitored by measuring the loss in

fluorescence over time (emission = 410 nm), upon the addition of 1.25 mM unlabeled ATP at time = 0. A representative experiment is shown (N=3). The concentrations of TgADF used, given in order of appearance, were 0, 0.1, 0.25, 1, 2, 0.5, 10 and 20 μM (represented by a shaded triangle to the right of the graph). *AU* = arbitrary units. *B*, Effect of TgADF on the nucleotide exchange rate of *T. gondii* actin (TgACT) monomers. The rate of nucleotide exchange on TgACT monomers was measured in the presence of varying concentrations of TgADF (0-20 μM). Experiments were done as in (A). A representative experiment is shown (N=3). The concentrations of TgADF used, given in order of appearance, were 0.25, 0, 0.5, 1, 2, 10 and 20 μM (represented by a shaded triangle to the right of the graph). *C*, Plot of the observed rate constants for nucleotide exchange on TgACT and RbACT monomers in the presence of varying concentrations of TgADF. Rate constants were derived from the initial reaction rates calculated from curves similar to those shown in (A) and (B) and plotted against TgADF concentration. The data were fit using first-order exponential decay kinetics and represent the averages of two (RbACT), or three (TgACT), independent experiments (mean ± S.E.M.).

Table S1. Primer sequences used to generate plasmid constructs

Proteins	Plasmid	Primer Sequence	
		Forward (5'->3')	Reverse (5'->3')
TgADF WT	pET16b+/ TgADF	GGG AAT CCA TAT GGC GTC CGG AAT GGG TGT TG	GAC GGA TCC TTA CGC GAG GGG TGC GAG GTC GC
TgADF G66K	pET16b+/ TgADF G66K	GCC GAT TCG GCG TGT ACG ACT GCA AGA ACA AGA TCC AGT TCG TTC TTT G	CAA AGA ACG AAC TGG ATC TTG TTC TTG CAG TCG ATC ACG CCG AAT CGG C
TgADF G66R	pET16b+/ TgADF G66R	CGG CGT GTA CGA CTG CCG CAA CAA GAT CCA GTT C	GAA CTG GAT CTT GTT GCG GCA GTC GAT CAC GCC G
TgADF-t	pET16b+/ TgADF-SpCOF-t	GGG AAT CCA TAT GGC GTC CGG AAT GGG TGT TG	GAG GAT CCT ACT TAC GAG TAA CCT TCT CAA GAA CCG CGA GGG GTG CGA GGT CGC
TgADF S3A	pET16b+/ TgADF S3A	GAA GGT CGT CAT ATG GCG GCC GGA ATG GGT GTT GAC G	CGT CAA CAC CCA TTC CGG CCG CCA TAT GAC GAC CTT C
TgADF S3C	pET16b+/ TgADF S3C	GAA GGT CGT CAT ATG GCG TGC GGA ATG GGT GTT GAC G	CGT CAA CAC CCA TTC CGC ACG CCA TAT GAC GAC CTT C
TgADF S3E	pET16b+/ TgADF S3E	GAA GGT CGT CAT ATG GCG GAG GGA ATG GGT GTT GAC GAG	CTC GTC AAC ACC CAT TCC CTC CGC CAT ATG ACG ACC TTC

Chapter 4

Actin Depolymerizing Factor controls actin turnover and gliding motility in *Toxoplasma gondii*

PREFACE

The first complete draft of this chapter was written by Simren Mehta. Comments from L. David Sibley were incorporated into the final version presented here.

Experiments presented in Figures 6 and 7 were performed after the defense and added to this chapter. A revised version of this chapter was submitted for publication to Molecular Biology of the Cell.

ABSTRACT

Apicomplexan parasites utilize a unique process of actin-based motility termed gliding, to migrate across cell surfaces and to power invasion and egress from host cells. Gliding requires the polymerization of actin filaments within parasites, yet 98% of parasite actin is non-filamentous. Previous work suggests that the inherent instability of parasite actin filaments accounts for the absence of filamentous actin *in vivo*. In higher eukaryotes the Actin Depolymerizing Factor (ADF)/Cofilin family of proteins are essential for actin filament turnover. We have previously shown that *Toxoplasma gondii* ADF has strong actin monomer sequestering and relatively weak filament severing activities *in vitro*. To investigate the role of ADF *in vivo* we generated a conditional knockout. We found that suppression of ADF results in stable actin filaments in *T. gondii*. Parasites were unable to engage in sustained helical gliding and moved at dramatically diminished speeds. Severe defects in invasion and egress were also observed. Our data suggests that ADF is essential for the rapid turnover of parasite actin filaments *in vivo*, and this is required for productive gliding motility.

INTRODUCTION

Apicomplexan parasites utilize a unique process of actin-based motility termed gliding to move across cell surfaces, cross tissue barriers, and enter and exit host cells. Gliding differs from other types of motility in that it does not involve any external appendages such as cilia and flagella, and does not cause any change in shape of the parasite (Sibley, 2004). Instead, gliding is mediated by the translocation of adhesins along the surface of the parasite, via connection to the parasite's actomyosin cytoskeleton (Sibley, 2004).

Studies with cytochalasin D demonstrate that parasite actin filaments are essential for gliding motility and host cell invasion, but are not required for host cell attachment (Dobrowolski and Sibley, 1996). However stabilization of actin filaments with jasplakinolide treatment results in the loss of coordinated parasite movements, and inhibits host cell invasion (Poupel and Tardieux, 1999; Wetzel et al., 2003), suggesting the requirement for precisely controlled polymerization and turnover of actin filaments for productive motility.

Despite the absolute requirement for actin filaments, 98% of actin in *T. gondii* is non-filamentous (Dobrowolski *et al.*, 1997; Pinder *et al.*, 1998). Consistent with this, actin filaments have not been visualized *in vivo* using phalloidin staining or conventional microscopy techniques (Shaw and Tilney, 1999). Filaments have only been captured by freeze-fracture electron microscopy of gliding parasites, which reveal the presence of short filaments just beneath the parasite plasma membrane (Wetzel *et al.*, 2003; Sahoo *et al.*, 2006). The transient nature of filamentous actin in *T. gondii* suggests the rapid assembly and disassembly of parasite actin filaments *in vivo*.

T. gondii has one actin allele that has 83% identity to vertebrate actin and is expressed throughout the parasite life cycle (Dobrowolski *et al.*, 1997). In comparison to conventionally studied actins from yeast or vertebrate cells, parasite actin filaments are inherently less stable *in vitro* (Schmitz *et al.*, 2005; Sahoo *et al.*, 2006), and this is predicted to arise from key molecular substitutions at the monomer-monomer interface along the filament (Sahoo *et al.*, 2006). This has led to the model that the absence of detectable F-actin in parasites is due to the inherent instability of parasite actin filaments. Consistent with this, only a limited set of actin regulatory proteins are found in apicomplexan parasites, and these are biased towards G-actin binding proteins (Baum et al., 2006; Schüler and Matuschewski, 2006). Although recent studies have demonstrated the conserved activities of many of these proteins *in vitro*, little is known about the mechanisms by which they regulate parasite actin dynamics *in vivo*.

The Actin Depolymerizing Factor (ADF)/Cofilin proteins are essential in most systems, and play critical roles in regulating cell migration and cell division (Bamburg, 1999; Ono, 2007). They function to regulate actin filament turnover by binding to the sides of filaments and increasing the twist of the filament, leading to filament severing (McGough *et al.*, 1997), and increasing the rate at which monomers are released from the pointed ends of filaments (Carlier et al., 1997). More recently a small subset of this family was shown to sequester actin monomers (Yamashiro et al., 2005).

ADF/Cofilin proteins are one of the few actin binding proteins conserved in apicomplexan parasites, where they are referred to as ADF (Baum et al., 2006). Most apicomplexan parasites have one ADF allele with the exception of *Plasmodium*, which has two alleles (Schuler *et al.*, 2005; Doi *et al.*, 2010). We have previously shown *in vitro*

that *T. gondii* ADF has strong monomer sequestering activity and relatively weak severing activity compared to the prototypical yeast cofilin (Mehta and Sibley, 2010). To investigate the role of TgADF *in vivo*, here we generated a conditional knockout parasite line and investigated the effect of ADF suppression on *T. gondii's* intracellular life cycle.

MATERIALS AND METHODS

Parasite culture

T. gondii tachyzoites were propagated by growth in monolayers of human foreskin fibroblasts (HFF) cultured in Dulbecco's Modified Eagles Medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 2 mM glutamine, 20 mM HEPES (pH 7.5), and 20 μg/ml gentamicin. Tetracycline-free fetal bovine serum (Hyclone; Logan, UT) was utilized when culturing the merodiploid and TgADF cKO parasite strains. Chloramphenicol (20 μg/ml) (Sigma-Aldrich, St. Louis, MO), phleomycin (5 μg/ml) (Invitrogen, San Diego, CA) and anhydrotetracycline (Atc) (1.5 μg/ml) (Clonetech, Palo Alto, CA) were added to the media as indicated.

Antibodies

TgADF was detected using a rabbit polyclonal antibody that was commercially generated (Covance Research Products, Inc., Denver, PA) against recombinant full length TgADF cloned with a C-terminal His6x tag in the pET22b+ vector (Novagen, Darmstadt, Germany). Protein expression was induced by the addition of 1 mM IPTG and the soluble fraction of TgADF was purified from *Escherichia coli* BL21 cells as previously described (Mehta and Sibley, 2010). Rabbit polyclonal antibodies to recombinant TgACT, expressed as a natively folded protein in baculovirus (Sahoo *et al.*, 2006), were also produced commercially. Aldolase was detected using rabbit polyclonal antisera to TgALD1 (Starnes *et al.*, 2006). The surface antigen SAG1 was detected using the mouse monoclonal antibody DG52.

Generation of the TgADF conditional knockout

The *TgADF* cKO was constructed using the tetracycline transactivator system (Meissner *et al.*, 2002). *TgADF* (GenBank accession number AAC47717) was PCR amplified to include a C-terminal HA9 tag (primer sequences given in supplemental Table S1) and cloned into the tetracycline regulated expression vector pTetO7Sag4 using the Mfe1 and Pac1 restriction sites, generating the plasmid pS4ADFHA (description of strains and plasmids given in supplemental Table S2). To provide selection, the *cat* gene (conferring chloramphenicol resistance) driven by the *SAG1* promoter was cloned into the Xba1 site on the plasmid backbone. This construct was transfected into a parasite line expressing the tetracycline transactivator (Meissner *et al.*, 2002), referred to here as the Tet-TA strain (Table S2), and after two rounds of drug selection stable transformants were single-cell cloned by limiting dilution. The resulting merodiploid clones were assayed by immunofluorescence and western blotting to confirm regulated expression of the TgADF-HA allele, and three clones were chosen to proceed with.

To generate the ADF knockout construct (pADFKO), the *ble* selectable marker conferring phleomycin resistance driven by the *SAG1* promoter, was flanked by genomic DNA sequence that lies 2 kb upstream and 2 kb downstream of the *TgADF* start and stop codons respectively (Table S1). As the frequency of homologous recombination is relatively low in *T. gondii*, a tandem YFP tag driven by the tubulin promoter, was placed downstream of the knockout cassette to provide a means for negative selection for double crossover events. pADFKO was linearized with the Apa1 restriction endonuclease and transfected into the chosen merodiploid clones. Phleomycin was used to select for stable transformants followed by fluorescence-activated cell sorting using a Dako MoFlo

(Carpinteria, CA) to select for YFP-negative parasites. Parasites were subsequently single cell cloned by limiting dilution and potential ADF cKO clones were screened by PCR using diagnostic PCR primer pairs 1-2 and 3 - 4 (Table S1).

Western blotting

Parasite lysates were resolved on 15% SDS-PAGE, transferred to membranes and probed with rabbit anti-TgADF or rabbit anti-aldolase antibodies, followed by a peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). Chemiluminescence with ECL Plus (GE Healthcare, Piscataway, NJ) was used for detection, and bands were quantified using an FLA-5000 phosphorimager (Fuji Film Medical Systems, Stamford, CT).

Immunofluorescence microscopy

For detection of ADF protein shutdown by immunofluorescence microscopy, parasites were pretreated with 1.5 μg/ml Atc for 24 h before infecting HFF monolayers grown on coverslips, followed by growth for an additional 24 h. Coverslips were fixed with 4% paraformaldehyde and permeabilized with 0.25% Triton X-100, before staining with anti-TgADF and anti-SAG primary antibodies followed by secondary antibodies conjugated to AlexaFluor 488 or AlexaFluor 594 (Invitrogen), respectively. Images were acquired using a Zeiss Axioskop 2 MOT Plus microscope equipped with a 63X 1.3 numerical aperture lens and an AxioCam Mrm camera (Carl Zeiss, Inc.; Thornwood, NY). The same exposure time controlled by Axiovision v4.2 software was used for imaging different strains.

For detection of F-actin in parasites, freshly harvested parasites were resuspended in HHE buffer (Hanks Balanced Salts (Sigma), 1 mM EGTA and 10 mM HEPES, pH 7.4) and parasites were allowed to glide on BSA (50 μg/ml) coated coverslips for 15 minutes at 37°C, before cells were fixed with 4% paraformaldehyde and permeabilized with 0.25% Triton X-100. Parasites were stained with rabbit anti-TgACT and mouse anti-SAG1 antibodies, followed by AlexaFluor 488 and AlexaFluor 594 conjugated secondary antibodies, respectively. Samples were mounted in Pro-Long Gold anti-fade reagent containing DAPI (Molecular Probes), and images were acquired as z-stack series (0.255 μm steps) using a Zeiss Axioskop 2 MOT Plus fluorescence microscope equipped with a 100X, 1.4 NA Plan Apochromat lens and a Axiocam Mrm monochrome camera (Carl Zeiss Inc.). Images were deconvolved in Axiovision v.4.5 software using the nearest-neighbor algorithm.

Electron microscopy

Freshly harvested parasites were resuspended in HHE buffer, incubated for 15 min at 37°C, and fixed in 2% paraformaldehyde/2.5% glutaraldehyde (Polysciences Inc., Warrington, PA) in 100mM phosphate buffer, pH 7.2 for 1 h at room temperature. Samples were washed in phosphate buffer and postfixed in 1% osmium tetroxide (Polysciences Inc.) for 1 h. Following extensive washing in distilled water, samples were en bloc stained with 1% aqueous uranylacetate (Ted Pella Inc., Redding, CA) for 1 h. Samples were dehydrated in a graded series of ethanol and embedded in Eponate 12 resin (Ted Pella Inc.). Sections of 95 nm were cut with a LeicaUltracut UCT ultramicrotome (Leica Microsystems Inc., Bannockburn, IL) and stained with uranyl acetate and lead

citrate. Samples were viewed and photographed on a JEOL 1200 EX transmission electron microscope (JEOL USA, Peabody, MA) and images adjusted linearly using Adobe Photoshop v8.0.

Plaque assay

Plaque assays were performed as previously described (Roos *et al.*, 1994). Confluent monolayers of HFF cells grown in six-well plates were infected with 200 parasites per well, supplemented with 1.5 μg/ml Atc as indicated. After 18 h of infection extracellular parasites were removed and Atc added back to the indicated wells at a concentration of 1 μg/ml. Monolayers were fixed with cold methanol 8 days after infection and stained with 0.1% crystal violet. Experiments were repeated three times with triplicate wells per experiment.

Invasion assay

Two-color invasion assays were performed as previously described (Buguliskis *et al.*, 2010) with minor modifications. Parasites were treated in the presence of 1.5 μg/ml Atc for 66 h unless otherwise noted. For the ADF cKO +Atc, five times the amount of parasites were used to reinfect host cells as compared to the other strains as these parasites demonstrated an invasion defect even after 24 h of Atc treatment. Freshly egressed parasites were resuspended in invasion media (low bicarbonate DMEM, 20mM HEPES, 3% FBS, pH7.4) and $5x10^5$ parasites were added to subconfluent host cell monolayers seeded on coverslips. A brief 1 min spin at 1000 rpm was used to deposit parasites onto the cell monolayer and parasites were allowed to invade for 20 min at

37°C. Non-attached parasites were removed by washing with invasion media and cells were fixed with 2.5% paraformaldehyde for 15 min at 4 °C. Extracellular parasites were detected with the DG52 mouse monoclonal antibody to SAG1, directly conjugated to AlexaFluor 594. After permeabilization with 0.25% Triton X-100, all parasites were detected with the DG52 primary antibody, followed by the AlexaFluor 488 secondary antibody. Extracellular and intracellular parasites and host cell nuclei were counted from 5 fields per coverslip from three coverslips, in two independent experiments. Data is plotted as the average number of parasites per host cell as well as the percent of total parasites invading host cells. Values represent the mean ± SD from 6 samples.

Video microscopy

Time-lapse video microscopy was conducted using a Zeiss Axiovert phase-contrast microscope equipped with a temperature-controlled stage (Medical Systems, Corp., Greenvale, NY) to maintain 37°C incubation. Images were collected under low-light illumination using an intensified CCD C2400 camera (Hamamatsu Photonics K.K., Hamamatsu City, Japan) at 40X magnification, and images were acquired using OpenLab software (Improvision, Waltham, MA) to control the shutters and camera.

Ionophore-induced egress

HFF cells that had previously been seeded on glass-bottom culture dishes (MatTek Corp., Ashland, MA) were infected with parasites that had been treated with Atc for one cycle, and allowed to grow for an additional 24 h. Extracellular parasites were removed and the media was replaced with Ringers solution containing 1% fetal bovine serum. Dishes were

allowed to equilibrate on the microscope heated stage before the addition of 2 μM calcium ionophore A23187 (Calbiochem). Recording was started immediately and egress was monitored over 10 min with a 1 s delay between frames and exposure times of approximately 110 ms.

Quantitation of egress kinetics was based on the analysis of time-lapse videos. Approximately 30 vacuoles containing 2-8 parasites per vacuole from two independent experiments were analyzed per strain. The time taken to motility, egress of the first parasite from the vacuole, and complete egress of all the parasites from the vacuole, was recorded. In cases where not all the parasites left the vacuole, the time at which the last parasite egressed from the vacuole was recorded.

Analysis of gliding motility

Freshly egressed parasites were harvested and resuspended in Ringer's media (155 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 3 mM NaH_2PO_4, 10 mM HEPES, 10 mM glucose) and added to glass-bottom culture dishes that had previously been coated with 50 μg/ml bovine serum albumin and washed with phosphate buffered saline. Parasites were allowed to settle for 2-3 min before recording was started. Time-lapse movies of at least 75 frames were collected, with exposure times ranging from 60 –150 ms per frame and a 1 s delay between frames.

For quantifying the type of motility, composite images were imported into ImageJ (http://rsb.info.nih.gov/ij/) and the Cell Counter plugin was used to count the total number of parasites, and the associated motility phenotype was scored by the observer.

Helical, circular, and twirling movements were defined as previously described (Hakansson, 1999). Briefly, helical gliding referred to a clockwise corkscrew motion that resulted in net forward migration of the parasite. Circular gliding occurred when the parasite lay on its right-side and movement traced the arc of a counterclockwise circle. Twirling referred to vertically oriented parasites that were engaged in a clockwise spinning motion while remaining attached to the substratum at the posterior end. Non-motile parasites were classified as parasites that were attached to the substratum but were either stationary or essentially remained in the same spot over the time-period analyzed (but did not engage in twirling).

New motility behaviors that were predominantly seen in the ADF cKO + Atc included partial patterns that were stalled or very slow. Half-circle and half-helix movements referred to parasites engaged in directional movement that resembled circular or helical patterns but the parasite did not complete the full movement within the 75-frame time-period analyzed. Short movement was classified as directional movement captured within the 75-frame time-period in which the displacement was not sufficient to determine the type of motility the parasite was engaged in. The half-circle, half-helix and short movements were combined into the short category for graphing purposes in Figure 5A. Uncoordinated movement was defined as movement involving frequent reversals of direction and little net migration, and was observed as a side-to-side rocking behavior, or a tumbling/rolling movement, similar to what has previously been described for jasplakinolide treated parasites (Wetzel *et al.*, 2003). Values for quantifying the type of motility were based on the quantification of 6 movies per condition, taken from two independent experiments. An average of 85 parasites were counted per video.

To calculate the speed of motility, parasites were chosen and tracked using the ImageJ particle tracker plugin (https://weeman.inf.ethz.ch/ParticleTracker). Tracks were individually inspected for accuracy and to define the beginning and end of each trajectory. Speed was calculated by measuring a parasite's displacement and dividing by the time taken for that displacement. Values are based on the quantitation of at least 20 parasites per condition taken from at least three movies.

Actin sedimentation assay

Freshly harvested parasites were resuspended in actin stabilization buffer (25 mM HEPES, 10 mM EGTA, 2 mM $MgCl_2$, 125 mM KCl) and incubated at 37^{o}C for 30 min. Reactions were supplemented with a final concentration of 1% Triton X-100, DNAse1 (10 μg/ml), protease inhibitor cocktail (E64, 1 μg/ml; AEBSF, 10 μg/ml; TLCK, 10 μg/ml; leupeptin, 1 μg/ml), and 10% glycerol, and parasites were lysed for 1 h at room temperature. Membrane ghosts were removed by low speed centrifugation at 1000 ***g*** for 2 min and supernatants were centrifuged at 350,000 ***g*** for 1 h at 4^{o}C (TL100 rotor, Beckman Optima TL Ultracentrifuge, Beckman Coulter, Fullerton, CA). Pellet samples were washed once with actin stabilization buffer, proteins were precipitated from the soluble fraction with acetone, and equal fractions of pellet and supernatant were resolved by 15% SDS-PAGE. Actin was detected by western blot analysis with a rabbit anti-*T. gondii* actin antibody as described above.

Statistics

All results are presented as mean values ± SD or SEM. Unpaired, equal variance, two-

tailed Student's *t* tests were used to determine the statistical significance of differences observed between indicated groups for parametric comparisons. The Mann-Whitney test was used for non-parametric comparisons.

RESULTS

Generation of a conditional knockout of TgADF

To examine the function of TgADF *in vivo* we generated a conditional knockout using the previously described Tet- transactivator system (Meissner *et al.*, 2002). *TgADF* was cloned with a C-terminal epitope tag under the control of the *SAG4* tetracycline regulatable promoter (Meissner *et al.*, 2002) and introduced into the parental Tet-TA strain of *T. gondii*, which harbors the Tet-transactivator (Meissner *et al.*, 2002) (Fig 1A). This generated a merodiploid line that expressed the endogenous *ADF* gene and the tetracycline regulatable *ADF* allele. To create the ADF conditional knockout (ADF cKO), the endogenous *ADF* gene was replaced by the *ble* selectable marker (Messina *et al.*, 1995) flanked by the untranscribed genomic sequence 2 kb upstream and downstream of the *ADF* gene (Fig 1A). Correct integration of the *ble* gene at the endogenous *ADF* locus was confirmed by PCR analysis using primers placed within the *ble* resistance cassette (Fig 1A, cKO diagnostic primers 2 and 3), combined with primers homologous to the genomic sequence in the *ADF* locus outside of the sequence used in the knockout cassette (Fig 1A, cKO diagnostic primers 1 and 4). A PCR product of 2 kb with both primer pairs 1-2 and 3-4, indicated the correct integration of the knockout cassette at the endogenous *ADF* locus (Fig 1B). Only one ADF cKO clone was isolated out of 450 clones screened (Fig 1B), indicating that a conditional knockout of ADF was relatively difficult to obtain. Deletion of the endogenous *ADF* gene was confirmed at the protein level by western blot analysis with anti-ADF antibodies (Fig 1C). Consistent with the loss of endogenous *ADF*,

only the tagged ADF protein was detected in the ADF cKO, and was expressed at 78% of the endogenous ADF level in the parental Tet-TA line (Fig 1C).

Suppression of ADF protein expression

To determine the degree to which ADF was suppressed in the ADF cKO, parasites were grown in the presence of anhydrous tetracycline, Atc (a non-cytotoxic derivative of tetracycline), and examined by immunofluorescence using antibodies against TgADF. In untreated parasites, ADF was dispersed evenly throughout the parasite cytosol (Fig 2A). After growth in Atc for 48 h, ADF expression was undetectable in ADF cKO parasites while the merodiploid strain still expressed ADF (Fig 2A). To examine the kinetics and extent of ADF shutdown, western blot analysis was used to quantify ADF protein levels in parasite lysates after treatment with Atc for varying time intervals (Fig 2B). Probing ADF cKO parasites with anti-TgADF antibodies revealed >80% of the protein was turned off in the first 24 h, relative to ADF levels in untreated parasites. After 48 h of Atc treatment, <5% of starting ADF levels remained.

Effect of ADF suppression on plaquing and invasion

The effect of ADF suppression on the intracellular life cycle of *T. gondii* was examined using a plaquing assay (Fig 3A). This assay reflects the ability of parasites to invade, replicate, egress and disperse from the original infected cell. In the Tet-TA and merodiploid strains, approximately the same number of plaques were formed in the absence or presence of Atc (although plaques were slightly smaller due to prolonged growth in Atc) (Fig 3A). Strikingly, although the ADF cKO grew as well as control

strains in the absence of Atc, no plaques were detected when ADF cKO grown in the presence of Atc. To determine which steps in the cycle were disrupted by ADF suppression, we examined each process in turn. Intracellular growth was examined by counting the number of parasites per vacuole at 12, 24 and 36 h post-infection (Taylor *et al.*, 2006), when parasites were grown in the absence or presence of Atc for the same amount of time. No differences in parasite growth were observed under these conditions (data not shown).

To investigate the ability of ADF cKO parasites to invade host cells, a two-color invasion assay was used to differentially stain intracellular and extracellular parasites via antibody staining (Buguliskis *et al.*, 2010). After 66 h of growth in Atc, the attachment and invasion of the merodiploid was comparable to untreated parasites (Fig 3B). ADF cKO parasites displayed a significant decrease in the total number of parasites per host cell even when grown in the absence of Atc, indicating that expression of the tagged allele is not fully wildtype, even though it is sufficient to support growth. Regardless of this modest defect, treatment of the ADF cKO with Atc led to a 57% decrease in the number of intracellular parasites per host nuclei (Fig 3B). Similarly, when parasite invasion was examined as the percentage of parasites that had invaded after a 20 min invasion pulse, suppression of ADF resulted in a 56% drop in invasion over untreated ADF cKO parasites (Fig 3C).

Effect of ADF suppression on ionophore-induced egress from host cells.

To determine if ADF suppression affected egress by *T. gondii*, we used time-lapse video microscopy to examine parasite exit from mature vacuoles stimulated with calcium

ionophore A23187, as described previously (Endo et al., 1982). Parasites were monitored to determine the onset of motility, length of time for egress, and dissemination from the infected host cell. The merodiploid and untreated ADF cKO strains behaved similarly, initiating motility within the vacuole at approximately 1.5 min after ionophore stimulation (Fig 4A, B), and completing egress within the next 1.2 min (Table 1). In contrast, ADF cKO parasites treated with Atc were slower to initiate motility (mean time 2.7 ± 1.1 min (± SD)), and displayed temporal heterogeneity in the time required to first become motile (Fig 4A, B). Furthermore, even after motility was initiated, Atc treated ADF cKO parasites were slow to leave the vacuole, with one or more parasites still remaining inside the majority of vacuoles (24/33) after 10 min (Fig 4B, Table 1). After emerging from the vacuole, merodiploid and untreated ADF cKO parasites were highly motile, often moving quite far from the original vacuole (Fig 4A). In contrast, parasites in which ADF had been suppressed were relatively non-motile and remained close to the point of exit from the vacuole (Fig 4A).

Effects of ADF suppression on T. gondii gliding motility

To directly investigate the effect of ADF suppression on motility, we utilized time-lapse video microscopy to examine extracellular parasites gliding on glass coverslips. Parasites expressing ADF were able to undergo multiple rounds of helical motility whereas parasites in which ADF was suppressed, were severely impaired and unable to complete a single helical cycle within the same time period (Fig 5A, B). Similarly, ADF suppressed parasites were also unable to complete a single cycle of circular movement within the observation period (Fig 5A). Instead, ADF suppression resulted in slow, serpentine

movements that generated elongated trails. These behaviors were classified as short movements in the graphical representation in Fig 5A. Quantification of the speed of parasite movement revealed a slight reduction in the speed of both helical and circular gliding between the merodiploid strains and the ADF cKO grown in the absence of Atc (Fig 5C, Table 2), consistent with the partial defect seen in the invasion assays. Nonetheless, with ADF suppression both helical and circular speeds were further reduced by 64% and 48% respectively (Table 2). Interestingly, an increased frequency of uncoordinated movements was observed in parasites in which ADF had been suppressed (Fig 5A). Such behaviors were characterized by frequent reversals of direction leading to no net movement, and were observed as back and forth rocking motions, or uncoordinated movements including "rolling" (data not shown in thesis). These behaviors appeared similar to movements observed when parasites have been treated with the actin stabilizing agent jasplakinolide (Wetzel *et al.*, 2003).

ADF suppression leads to more stable actin in T. gondii

To examine if actin filaments are more stable in the absence of ADF, actin sedimentation assays were used to compare the ratio of unpolymerized and polymerized actin in parasite lysates (Fig 6A). Since parasite actin filaments are inherently less stable and do not form long filaments like conventional actins, parasite lysates were centrifuged at higher speeds, as has been previously reported (Mehta and Sibley, 2010). In parasites expressing ADF, approximately 15% of parasite actin sedimented when centrifuged at 350,000 ***g*** for 1 h (Fig 6A). In contrast, with ADF suppression, the proportion of pelletable actin was significantly increased, indicating that in the absence of ADF more parasite actin was

found in the polymerized form. To determine if actin filaments could be visualized in these parasites, immunofluorescence microscopy was used to localize actin in extracellular parasites undergoing gliding. Previous work has shown that actin in *T. gondii* typically localizes diffusely in the cytoplasm with a perinuclear concentration (Dobrowolski et al., 1997). In ADF cKO parasites expressing ADF, actin was found predominantly in the cytoplasm with a perinuclear concentration (Fig 6B, -Atc). However with ADF suppression actin was concentrated at the parasite periphery in a greater proportion of the parasites, often at one or both poles of the parasite (Fig 6B, +Atc). In addition, short cable-like structures were observed concentrated at the poles of the parasite (Fig 6B, middle), and these extended throughout the parasite cytoplasm tracing a spiral-like pattern (Fig 6B, right). Actin cables were also detected in multiple planes of the parasite, as depicted in the z-series in Fig 6C, occurring both beneath the parasite membrane (Fig 6C, right), and internally in the cytosol (Fig 6C, left).

To determine the nature of the actin-rich cables that occurred following ADF suppression we used transmission electron microscopy to examine the parasite ultrastructure. Consistent with previous reports (Wetzel *et al.*, 2003; Sahoo *et al.*, 2006), filamentous actin was not detected in parasites expressing ADF (Fig 7A, D). Strikingly, following ADF suppression an abundance of actin filaments were detected underlying the parasite's inner membrane complex (Fig 7B, C arrowheads), often resulting in an apical protrusion rich with actin filaments (Fig 7B, arrow). Similar filament-rich structures were also observed beneath the inner membrane complex at the basal end of parasites when ADF was suppressed (Fig 7E, F), and in cases where a basal protrusion was observed, it

was accompanied by a break in the inner membrane (Fig 7E). Collectively, these data demonstrate that stable actin filaments are formed in *T. gondii* in the absence of ADF.

DISCUSSION

Actin in apicomplexan parasites is almost all non-filamentous, despite the essential requirement for parasite actin filaments during gliding motility and invasion To investigate the function of ADF in *T. gondii*, we generated a conditional ADF knockout. Suppression of ADF resulted in greater than 50% reduction in invasion, and parasites were slow to egress from host cells. Detailed analysis of parasite motility revealed the inability to complete sustained helical motility and a 64% reduction in speed, with increased frequency of direction reversals. Ultrastructural analysis identified the accumulation of actin filaments beneath the parasite inner membrane complex, which often resulted in actin-rich projections at the apical and basal ends of the parasite. These data demonstrated that ADF is essential for regulating actin filament turnover in *T. gondii*, and that inherent instability of parasite actin filaments is not sufficient to account for the absence of filaments in *T. gondii*.

Suppression of ADF in *T. gondii* led to a significant impairment in host cell invasion. Interestingly, the 56% decrease in invasion with ADF suppression is similar to the 50% reduction in host cell invasion when parasites are treated with 0.05-0.1 μM jasplakinolide (JAS), which is also the half-maximal concentration of JAS at which inhibition of invasion and gliding is observed (Wetzel *et al.*, 2003). This concentration corresponds to the dose at which actin polymerization is first detected in parasites with JAS treatment (Wetzel *et al.*, 2003). Together these data suggest that the invasion defect observed with suppression of ADF, is due to the stabilization of actin filaments within the parasite.

Egress of parasites out of the parasitophorous vacuole is dependent on the activation of parasite motility within the vacuole and secretion of the perforin-like-protein plp1 (Kafsack *et al.*, 2009). With ADF suppression, most parasites initiated motility and began to leave the vacuole, although egress was incomplete, and the parasites that exited did not disperse. The partial defect in egress and invasion observed with ADF suppression contrasts the complete invasion and egress defects observed with suppression of profilin in *T. gondii* (Plattner *et al.*, 2008). Although profilin likely sequesters actin monomers similar to ADF, profilin is also predicted to promote filament polymerization and elongation in concert with formin proteins. An absence of filament polymerization with suppression of profilin could account for a complete inhibition of invasion and egress. In contrast, suppression of ADF is shown here to promote actin polymerization and/or filament stabilization. The short serpentine movements that result from ADF suppression may be sufficient to promote host cell entry and vacuolar egress, thus resulting in partial defects in both processes.

Frequent direction reversals seen with ADF suppression resemble the motility behaviors of JAS treated parasites (Wetzel *et al.*, 2003), suggesting the motility defects are due to stabilization of parasite actin filaments. Consistent with this, the ultrastructure of Atc treated ADF cKO parasites was surprisingly similar to JAS treated parasites (Shaw and Tilney, 1999), revealing the accumulation of parasite actin filaments beneath the inner membrane complex, and actin-rich projections at the apical and basal ends of the parasite. Based on the *in vitro* properties of *T. gondii* ADF (Mehta and Sibley, 2010), the increased abundance of parasite actin filaments is predicted to arise by two mechanisms. First, the absence of ADF sequestering activity likely increases the concentration of free

actin monomers in the parasite cytosol, resulting in the critical concentration being more easily attained, and shifting the actin equilibrium towards polymerization. Second, the absence of severing activity by ADF results in the accumulation of actin filaments once formed. Although it is difficult to tease apart the relative contributions of each activity *in vivo*, the effects of both activities are expected to be additive. Of particular importance, the accumulation of parasite actin filaments with ADF suppression indicates the intrinsic instability of parasite actin filaments is not sufficient to account for the absence of actin filaments *in vivo*.

In spite of the absolute requirement for parasite actin filaments during gliding motility and invasion, the presence of a more stable pool of F-actin with ADF suppression, was detrimental to both processes. There are a number of potential mechanisms that might explain these findings. A decrease in actin filament turnover is predicted to result in a diminishing pool of actin monomers, which would eventually limit new rounds of polymerization required for sustained motility. Alternatively, an overabundance of actin filaments might disrupt the efficient functioning of the myosin motor and translocation of surface adhesins, or result in increased membrane rigidity, preventing normal gliding motility. Additionally a denser actin cytoskeleton could affect substrate interactions required for normal motility. Consistent with this, recent work using transitional force microscopy to examine the motility of *Plasmodium* sporozoites revealed that stabilization of parasite actin filaments with JAS treatment decreased the adhesion strength of parasites to the cell surface, disrupting gliding motility (Munter *et al.*, 2009). Examining the mechanisms by which gliding motility is affected by the

presence of a more stable pool of actin filaments will be an important avenue for future research.

Overall, using a conditional knockout in ADF we have demonstrated the essential requirement for the regulated turnover of parasite actin filaments during gliding motility. Suppression of ADF in *T. gondii* led to stabilization of parasite actin filaments *in vivo*, indicating that intrinsic instability of parasite actin filaments is not sufficient to account for the absence of filaments *in vivo*. Stabilization of actin filaments was associated with a dramatic reduction in the speed of parasite motility, thereby impeding productive gliding, and also a loss of directionality in movement. The defects in motility were compounded over the parasite life cycle resulting in the inhibition of invasion, egress, and cell-to-cell spread, highlighting the importance of the correct functioning of the motility machinery for parasite survival. Ultimately the presence of high G-actin concentrations, unstable actin filaments and a process for regulated filament disassembly in apicomplexan parasites, highlights a system structured for rapid and efficient actin-based motility. Despite having a small cast of characters to orchestrate actin dynamics, apicomplexan parasites have devised multiple mechanisms to achieve rapid motility.

ACKNOWLEDGMENTS

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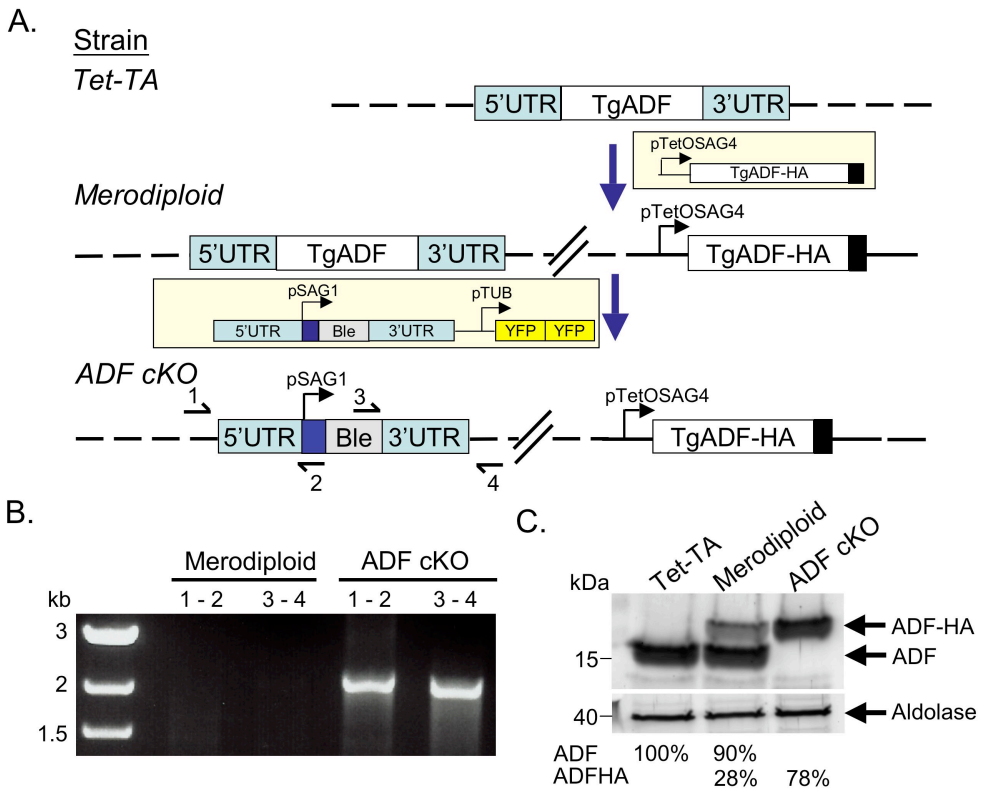

Figure 1. Generation of a conditional knockout of TgADF. (A) Diagram outlining the strategy used to generate the conditional knockout (cKO) of TgADF. The Tet-TA strain was transfected with a plasmid containing HA-tagged TgADF (TgADF-HA) under the control of the Tet-regulatable promoter pTetOSag4. This resulted in a merodiploid strain expressing 2 copies of TgADF. The endogenous TgADF gene was then replaced by transfecting in a plasmid containing the phleomycin resistance marker (ble) flanked by the ADF 5' and 3' untranslated regions (UTR). Double crossover events were screened for using tandem YFP (YFPYFP) as a negative selection marker. Diagnostic PCR primers 1-4 used to confirm the correct integration of the resistance marker are shown in the bottom panel. Dashed lines represent the chromosome of each strain. (B) PCR analysis demonstrating disruption of the TgADF locus. Diagnostic PCR primer pairs 1-2

and 3-4, depicted in (A) confirmed the successful replacement of the endogenous TgADF gene with the ble resistance cassette. Genomic DNA from the merodiploid strain was used as a negative control. (C) Western blot depicting the absence of endogenous TgADF protein and the relative expression level of the TgADF-HA allele in the ADF cKO. Parasite lysates were resolved by 15% SDS-PAGE and probed with anti-TgADF antibodies. Aldolase was used as a loading control. Phosphorimager quantitation of the ADF and ADF-HA bands is given below the blot as a percentage of the endogenous ADF protein level in the Tet-TA strain.

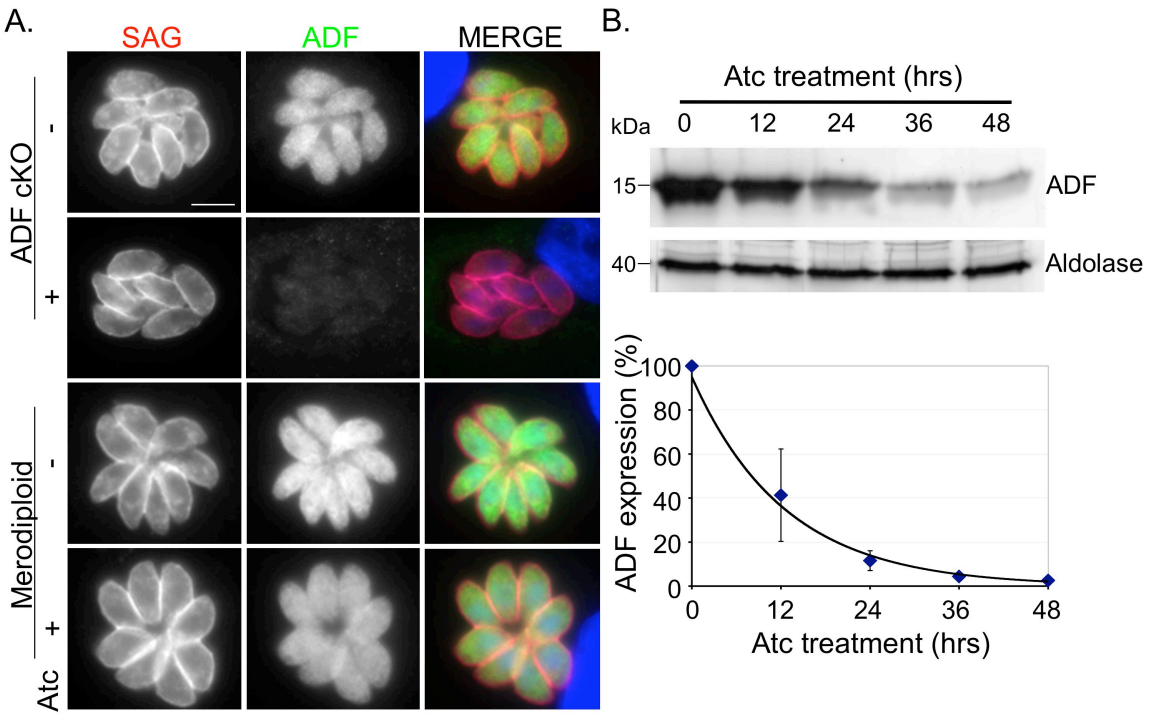

Figure 2. Suppression of ADF protein expression. (A) Immunofluorescence analysis of ADF repression in parasites cultured in the presence of 1.5 μg/ml Atc for 48 h. ADF (green) and SAG (red) were detected using rabbit anti-TgADF and mouse anti-SAG antibodies respectively. Scale bar, 5 μm. (B) Western blot analysis of the timecourse of ADF repression following Atc treatment. ADF cKO parasites were treated with Atc for 0 - 48 h and parasite lysates were probed with antibodies to TgADF, or aldolase as a loading control. Repression was quantified using phosphorimager analysis of the ADF bands and is represented as a percentage of ADF levels after 0 h of treatment. Values represent means ± SEM, N=3 experiments.

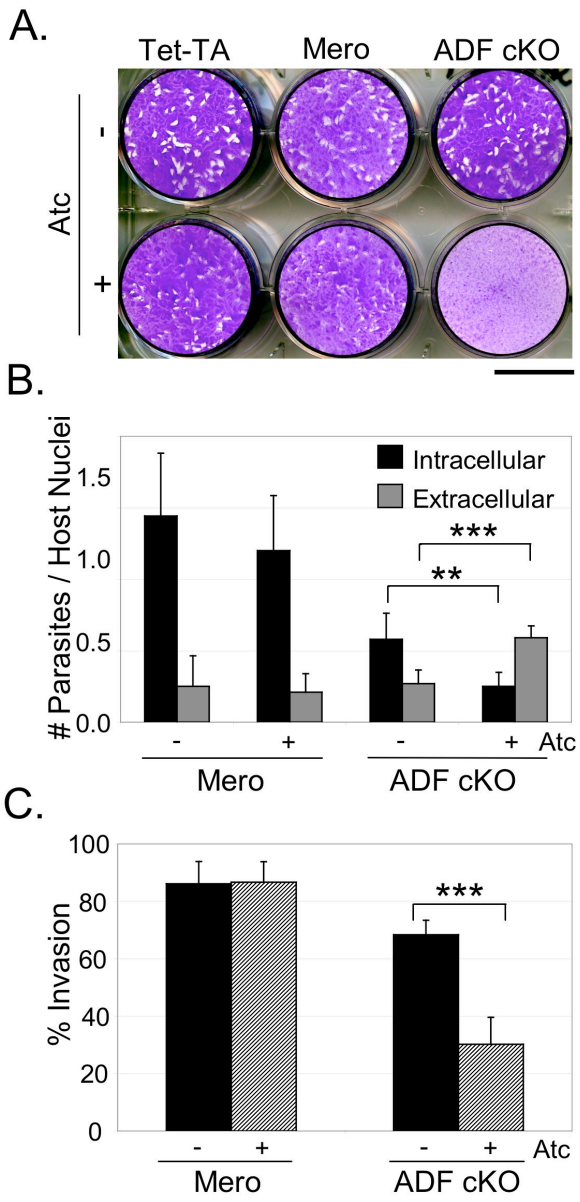

Figure 3. Effects of ADF suppression on plaquing and host cell invasion. (A) Plaque assay on fibroblast monolayers grown in the presence or absence of Atc for 8 days. Scale bar, 2 cm. (B) Parasite invasion of host cell monolayers as detected by the two-color immunofluorescence assay. Parasites were allowed to invade host cells for 20 min before being fixed and differentially stained for a parasite surface antigen to identify intracellular versus extracellular parasites. Data are represented as the number of

parasites invaded (intracellular), or attached (extracellular), per host cell nuclei. Values represent means ± SD, 3 samples from each of 2 experiments (N=6). ** $p \leq 0.005$, *** $p \leq 0.0001$. (C) The data from (B) is represented as the percentage of parasites out of the total number counted that had invaded host cells in the 20 min timeframe.

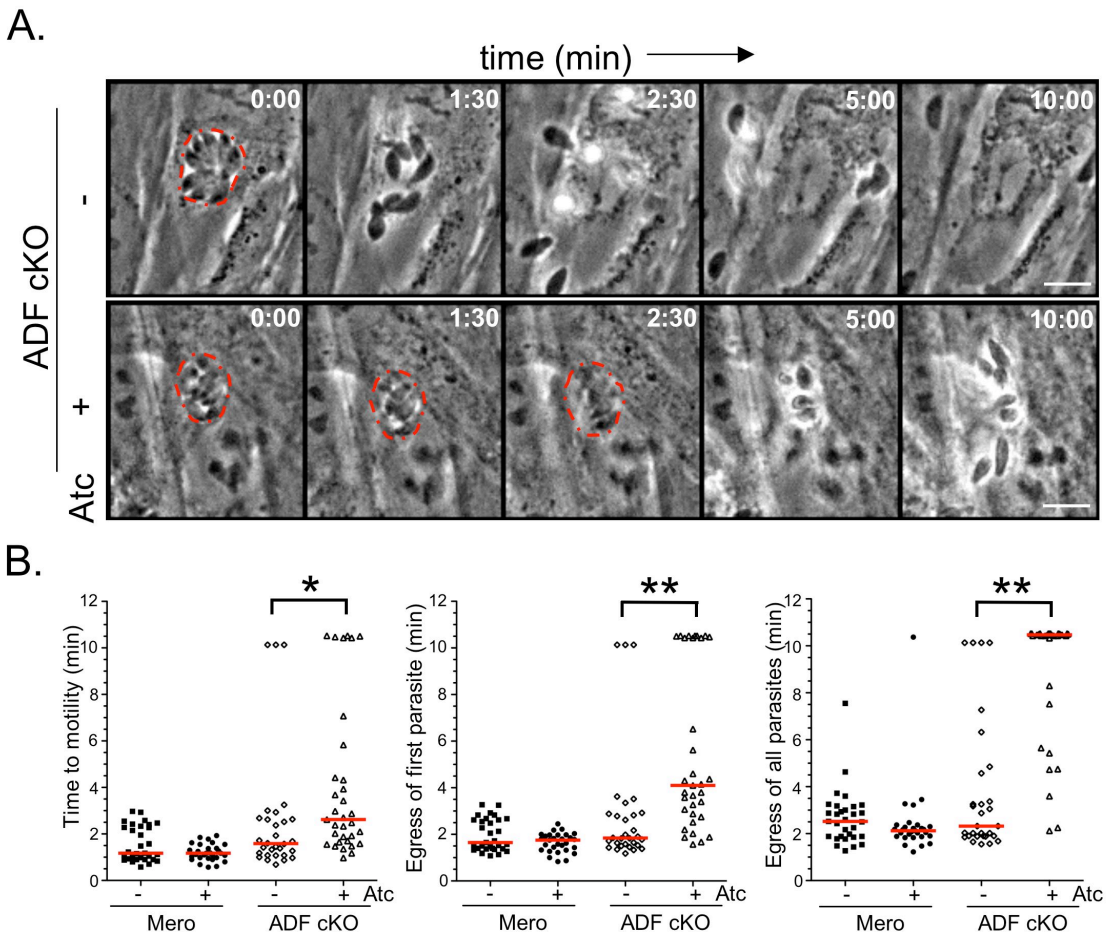

Figure 4. Effects of ADF suppression on egress from host cells. (A) Time-lapse video micrographs comparing ionophore-induced egress of ADF cKO parasites grown in the absence or presence of Atc. Infected host cells were stimulated with 2 μM calcium ionophore and parasite egress from the parasitophorous vacuole was monitored over time. Intact vacuoles are outlined in red, and the time elapsed after ionophore addition is indicated in minutes:seconds. Scale bar, 10 μm. (B) Quantitation of the kinetics of egress based on time-lapse video microscopy. The time taken for the activation of parasite motility within the vacuole (left), egress of the first parasite from the vacuole (middle), and egress of all of the parasites from the vacuole (right) is given in minutes after the addition of ionophore, for the merodiploid and ADF cKO strains grown in the absence or

presence of Atc. Parasites that did not leave the vacuole were assigned the value of the last time-point (10.5 min). At least 29 vacuoles were counted from two independent experiments. Bars represent the median time taken. Mann-Whitney test, * $p \leq 0.005$, ** $p \leq 0.0001$.

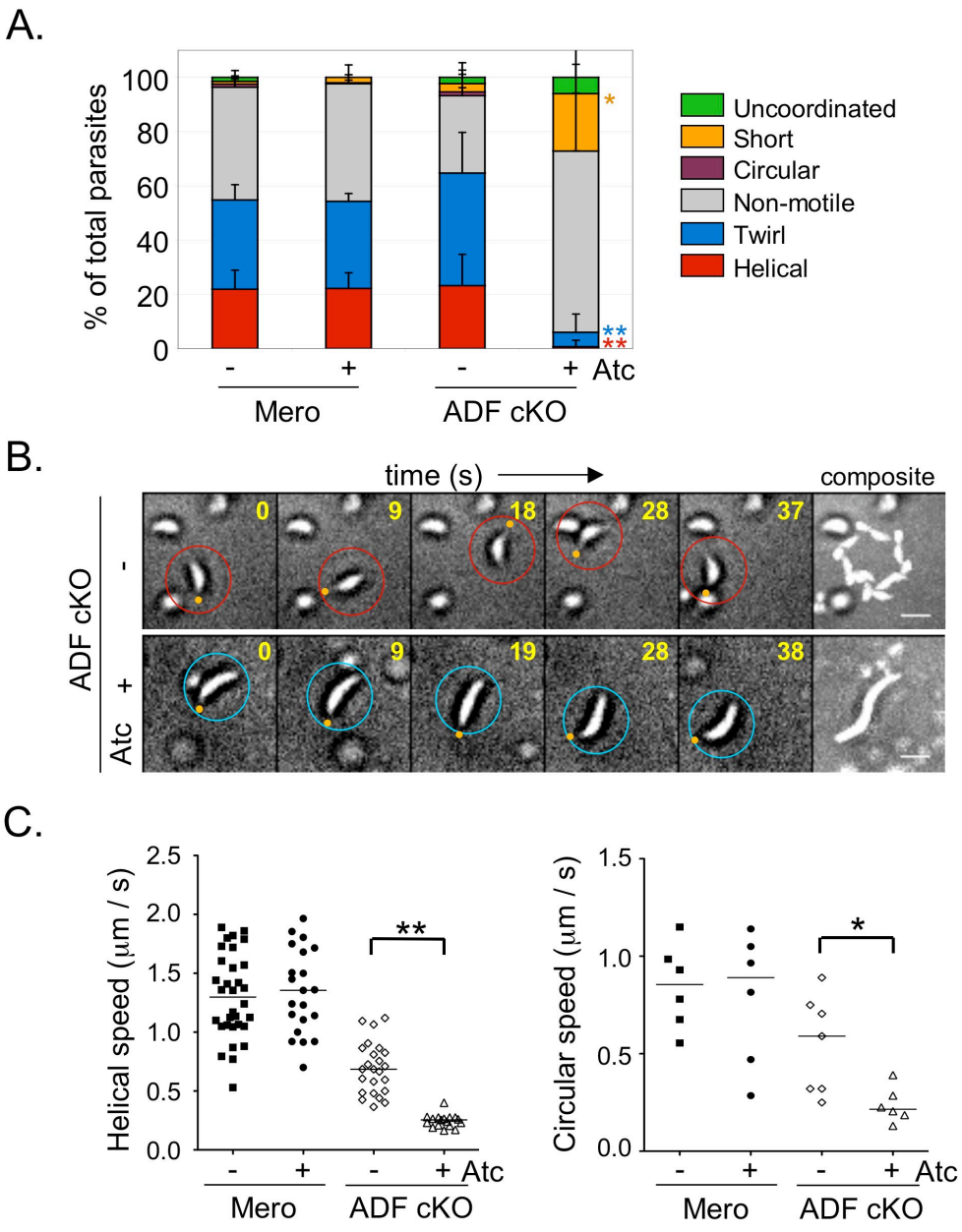

Figure 5. Effects of ADF suppression on gliding motility. (A) Quantitation of parasite motility as observed by time-lapse video microscopy. Freshly harvested parasites were allowed to glide on BSA-coated glass-bottom culture dishes and motility was documented by time-lapse video microscopy. The type of motility was categorized into the indicated classes as defined in the Methods, and the data is plotted as percent of total.

Values represent means ± SD, N=3 samples, from a representative of two similar experiments. Student's *t* test comparing ADF cKO - Atc vs. ADF cKO + Atc, * $p \leq 0.05$, ** $p \leq 0.01$. (B) Time-lapse video micrographs comparing helical gliding in ADF cKO parasites grown in the absence or presence of Atc. Shutdown of ADF protein expression results in slow gliding motility, depicted in the lower panel. The parasite of interest is highlighted with a circle (red, upper panel; blue lower panel), with the orange dot denoting the apical end of the parasite. The last micrograph in both panels represents a summation of the indicated parasite's movement over 45 seconds. Time elapsed is shown in seconds in the top right-hand corner. Scale bar, 5 μm. (C) Scatter plots depicting speeds of helical (left), and circular (right), gliding motility for the merodiploid and ADF cKO strains grown in the absence or presence of Atc. The distance migrated by parasites was tracked over time based on time-lapse movies. Helical gliding speeds were calculated from following the movements of at least 15 parasites for each strain, circular speeds were based on following at least 6 parasites per strain. Bars represent median values. Mann-Whitney test, *$p \leq 0.05$; **$p \leq 0.001$.

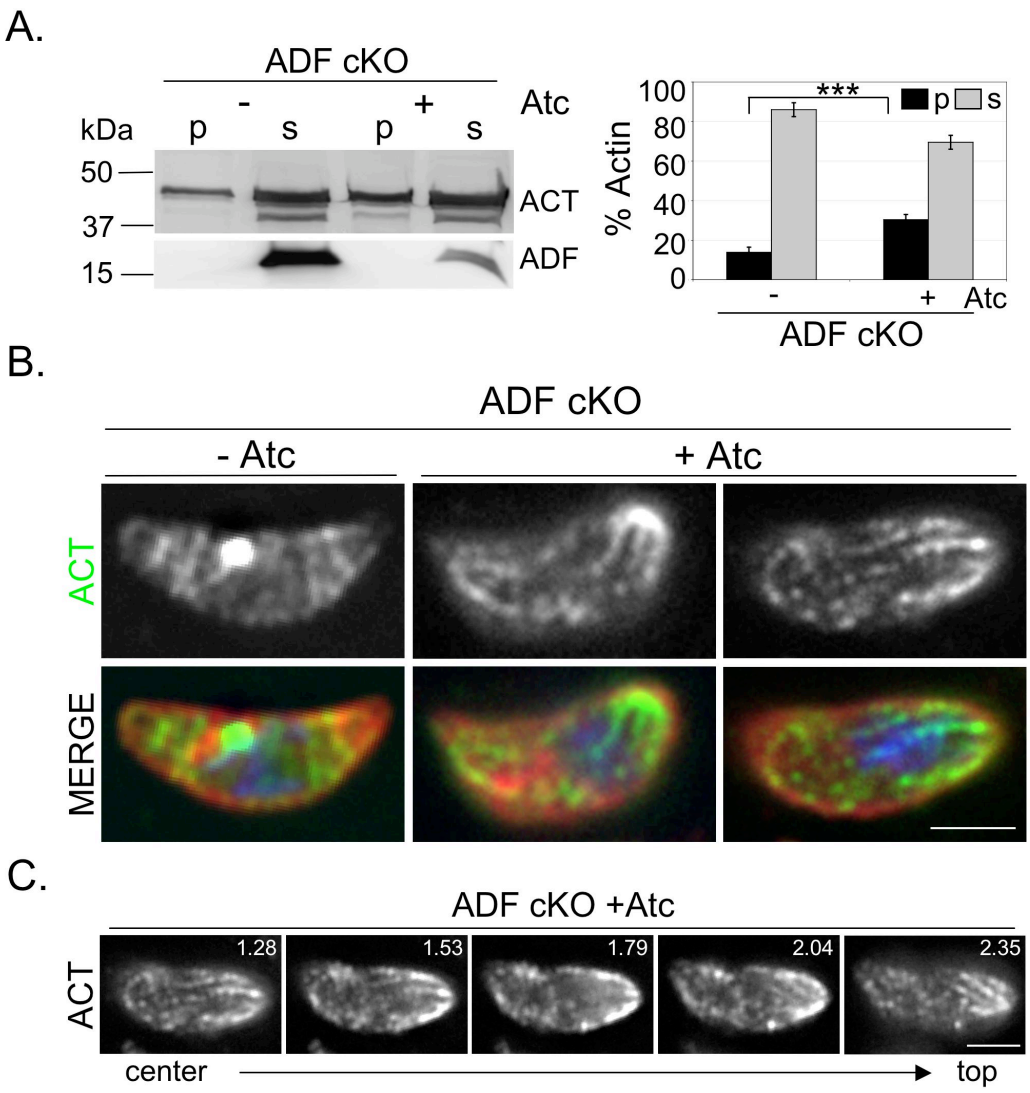

Figure 6. Effects of ADF suppression on actin filament polymerization in *T. gondii*. (A) Sedimentation analysis of F-actin in TgADF cKO parasites grown ± Atc for 66 h. Parasite lysates were centrifuged at 350,000 ***g*** for 1 h, and the pellet (p) and supernatant (s) fractions were analyzed by SDS-PAGE and quantitative western blotting using antibodies against *T. gondii* actin and ADF. Quantitation of the actin bands (ACT) by phosphorimager analysis is depicted on the right. Bars represent means ± SD (N=3 experiments), ***$p \leq 0.001$. (B) Immunofluorescence analysis of actin in ADF cKO parasites during gliding motility. ADF cKO parasites were grown ± Atc for 66 h,

harvested and allowed to glide on coverslips before cells were fixed and stained with antibodies against *T. gondii* actin (shown in green), and the parasite surface antigen SAG (shown in red). Suppression of TgADF resulted in cytoplasmic actin structures that were often concentrated at one or both poles of the parasite. Images were captured using wide-field microscopy and deconvolved using the nearest neighbor algorithm. The micrographs represent a single slice taken from a z-stack. Scale bar, 2 μm. (C) Z-series of actin staining of a single TgADF cKO parasite from (B) treated with Atc, depicting the presence of actin structures in multiple planes of the parasite. Depth of the depicted slice from the bottom of the parasite is given in the upper right hand corner in μm. Scale bar, 2 μm.

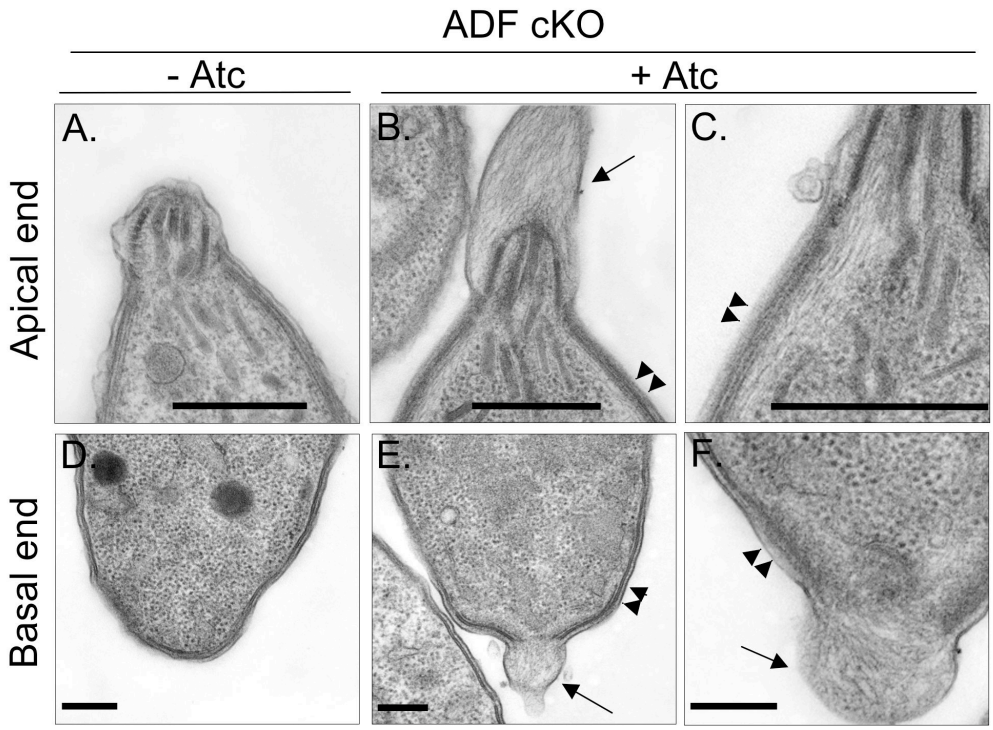

Figure 7. Ultrastructural analysis of actin filaments in *T. gondii* following ADF suppression. Electron micrographs of extracellular ADF cKO parasites after growth in the absence (-Atc) or presence of Atc (+Atc) for 66 h. The top panel depicts micrographs of the apical ends of parasites, while the bottom panel depicts the basal ends of different parasites. (A) Under normal conditions, actin filaments were not detected in parasites expressing ADF. Scale bar is 0.5 μm in all micrographs. (B) When ADF was suppressed, abundant actin filaments were detected in apical protrusions (single arrow), and just underneath the parasite plasma membrane (arrowheads). (C) Magnification of the region under the plasma membrane in a different parasite. (D) In ADF cKO parasites grown in the absence of Atc, no filaments were detected at the basal ends, and the parasite plasma membrane and inner membrane were intact. (E) Suppression of ADF resulted in actin filaments protruding from the basal end (arrow) accompanied by a gap in the inner membrane, and filaments were detected lining the inside of the plasma membrane

(arrowheads). (F) Magnification of the posterior protrusion (arrow) and submembranous actin filaments (arrowheads) in a different parasite.

Table 1. Characterization of ionophore-induced egress in *T. gondii*

Strain	Atc[a] treatment	Average time to egress (min)[b]	Number of vacuoles with incomplete egress[c]
Merodiploid	−	1.1 ± 0.4[d]	1 / 30
Merodiploid	+	1.2 ± 0.7	0 / 29
ADF cKO	-	1.2 ± 0.3	4 / 30[e]
ADF cKO	+	4.2 ± 0.6*	24 / 33[f]

[a] Anhydrotetracycline.
[b] Represents the average time taken from the initiation of motility to egress of the last parasite from the vacuole.
[c] Represents the number of vacuoles out of the total number of vacuoles analyzed in which all of the parasites did not egress in 10 min.
[d] Mean time ± SD.
[e] In 3 vacuoles, 0 parasites left the vacuole.
[f] In 8 vacuoles, 0 parasites left the vacuole.
* $p \leq 0.001$ Student's *t* test, ADF cKO - Atc vs. ADF cKO + Atc.

Table 2. Comparison of rates of *T. gondii* gliding motility

Strain	Atc[a] treatment	Type of motility			
		Helical (μm / s)	Circular (μm / s)	Short	
				Half-Helix (μm / s)	Half-Circle (μm / s)
Merodiploid	−	1.33 ± 0.13[b]	0.85 ± 0.09	—[c]	—
Merodiploid	+	1.38 ± 0.22	0.79 ± 0.14	—	—
ADF cKO	-	0.69 ± 0.05*	0.48 ± 0.14	—	—
ADF cKO	+	—	—	0.25 ± 0.01**	0.25 ± 0.01

[a] Anhydrotetracycline.
[b] Mean speed ± SEM.
[c] Behavior not observed.
* $p \leq 0.05$, Student's *t* test ADF cKO - Atc vs. Merodiploid + Atc.
** $p \leq 0.005$, Student's *t* test ADF cKO - Atc vs. ADF cKO + Atc.

Table S1. Primer sequences used to generate plasmid constructs

Primer	Use	Forward (5'->3')	Reverse (5'->3')
TgADFHA	To amplify *TgADF* with a C-terminal HA9 tag	GCG TAC AAT TGC CTT TTT CGA CAA AAT GGC GTC CGG AAT GGG TGT TG	CGG TTA ATT AAG CGT AAT CTG GGA CGT CGT ATG GGT ACG CGA GGG GTG CGA GGT CGC CC
TgADF 5' UTR	To amplify the 5' UTR of TgADF	GAC GGG CCC GAG AGT TCG AGC AGC CAC AGG	GGA CGG ACC GCT TGA CTG GAG TTT CGA GGA AAA
TgADF 3' UTR	To amplify the 3' UTR of TgADF	GGC TTA ATT AAA AAC TTC GTG AAA CGC GCG ACG	GGA CTA GTG CCG CAG AAA GGA AAA CTA GGC
cKO diagnostic primers 1 - 2	Diagnostic PCR primers to screen for ADF cKO	GCC GTA GGA CAG ATT GGT GT	GTG TGG GAA GTT TCG TTC GT
cKO diagnostic primers 3 - 4	Diagnostic PCR primers to screen for ADF cKO	ATT GCT TGC AGG CAT CTC TT	CCG TTG CAT ACT GCG AGA TA

Table S2. Description of *T. gondii* strains and plasmids used in this study

Parasite Line	Plasmid	Genotype	Paper Nomenclature	Citation
TAT-I		*TgADF$_e$*	Tet-TA	Meissner *et. al*
TAT-I: Merodiploid ADF	pS4ADFHA	*TgADF$_e$*/ *TgADF-HA*	Merodiploid	This study
TAT-I: TgADF conditional KO	pADFKO	- / *TgADF-HA*	ADF cKO	This study

Chapter 5

Conclusions and Future Directions

This chapter was composed entirely by Simren Mehta. Comments from L. David Sibley were incorporated into the final version presented here.

CONCLUSIONS

Apicomplexan parasites utilize a unique process of substrate-dependent motility termed gliding that allows them to move across host cell surfaces and invade cells at rapid speeds. Despite both gliding and invasion being critically dependent on the parasite's actin cytoskeleton, little is known about how parasite actin is assembled or regulated, and how this influences motility. Gliding is thought to require rapid cycles of filament assembly and disassembly, yet only a limited set of the conserved actin binding proteins are found in apicomplexan parasites (Baum *et al.*, 2006; Schüler and Matuschewski, 2006). In higher eukaryotes, the Actin Depolymerizing Factor (ADF)/Cofilin (AC) family of proteins are thought to be responsible for promoting the high rates of filament turnover observed *in vivo*, although they can interact with actin in a variety of ways (Bamburg, 1999). All apicomplexan parasites examined thus far encode for at least one copy of ADF in their genome (Baum *et al.*, 2006; Schüler and Matuschewski, 2006). In this thesis, I sought to determine the role of ADF in regulating actin dynamics in *T. gondii*. To do this, I first defined the mechanisms by which TgADF interacts with actin *in vitro*, for both *T. gondii* actin and the well-studied rabbit non-muscle actin. Subsequently, I tested the role of TgADF *in vivo* and found that TgADF is essential for regulating *T. gondii* gliding motility, and plays an important role in a number of steps in the parasite life cycle.

TgADF was found to be extremely potent at causing the net disassembly of actin filaments, resulting in the almost complete disassembly of actin filaments in actin sedimentation assays (Chapter 3). This is in sharp contrast to most members of the AC family (Carlier *et al.*, 1997; Maciver *et al.*, 1998; Pope *et al.*, 2000). As this assay

measured the net sum of a number of different possible interactions between TgADF and actin, the individual interactions were dissected using more specific assays. Two main ways in which TgADF functionally interacts with actin were identified (Chapter 3). Firstly, TgADF sequesters actin monomers and secondly, it severs actin filaments. TgADF did not nucleate the polymerization of actin filaments, as has been described for some members of the AC family (Andrianantoandro and Pollard, 2006), and in contrast, inhibited the polymerization of actin monomers. Thus, it is unlikely that TgADF has a role in nucleating actin filaments in the parasite. Recently it was demonstrated that the *Plasmodium* formin1 protein can nucleate the polymerization of heterologous actin *in vitro* (Baum *et al.*, 2008). As formin1 orthologues are found in the other apicomplexan parasites (Baum *et al.*, 2006; Schüler and Matuschewski, 2006), it is likely that formin1 functions to promote filament nucleation in apicomplexan parasites. Similar to other AC proteins (Nishida, 1985; Hawkins *et al.*, 1993; Hayden *et al.*, 1993; Andrianantoandro and Pollard, 2006), but different from the *Plasmodium* ADF homologue PfADF1 (Schuler *et al.*, 2005), TgADF was found to inhibit nucleotide exchange on actin monomers. This suggests that the lack of this activity in PfADF1 may be specific to PfADF1, rather than being a general feature of the apicomplexan ADF proteins.

TgADF was found to be a potent actin monomer sequestering protein *in vitro* (Chapter 3). This was identified by its ability to strongly inhibit the steady state polymerization of high concentrations of both *T. gondii* actin and rabbit actin using light scattering assays. This was further supported by calculating the affinity of TgADF for ATP-actin monomers to be approximately 0.8 μM, based on the inhibition of nucleotide exchange on TgACT monomers in the presence of TgADF (Chapter 3). The affinity of

TgADF for ADP-G-actin has yet to be determined. The moderate affinity of TgADF for ATP-actin monomers is in contrast to most AC family members, which typically bind to ADP-G-actin with an affinity of 0.13 μM, but have a weak affinity for ATP-G-actin of ~ 7 μM (Didry *et al.*, 1998; Chen *et al.*, 2004). The high affinity binding of AC proteins to ADP-bound actin is thought to provide a mechanism for selectively disassembling older filaments, which in contrast to young filaments, have hydrolyzed the bound nucleotide (Maciver *et al.*, 1991). Like other AC family members, TgADF is also predicted to have a higher affinity for ADP-actin. It would be important to experimentally determine this value to know if TgADF might also function to selectively disassemble older filaments. As ATP is typically in excess in the cell, most of the G-actin in the cell is expected to be bound to ATP (Rosenblatt *et al.*, 1995). Due to their low affinity for ATP-actin, AC proteins are typically thought not to function as monomer sequestering proteins (Chen *et al.*, 2004). However a small subset of AC proteins (Unc60A, embryonic chick ADF) inhibit the steady-state polymerization of actin, and like TgADF, have affinities of ~ 1 μM for ATP-G-actin, suggesting that they also sequester actin monomers (Chen *et al.*, 2004; Yamashiro *et al.*, 2005). Interestingly, these AC proteins are expressed in embryonic cells, a cellular environment undergoing large-scale cytoplasmic remodeling (Abe and Obinata, 1989; Ono *et al.*, 2003). A large proportion of actin in chicken skeletal muscle embryonic cells has been shown to be maintained as G-actin (Shimizu and Obinata, 1986), and this is thought to be generally true in embryonic cells, presumably allowing for the dynamic assembly of actin filaments. Thus AC proteins that are expressed in highly dynamic cellular environments appear to have monomer sequestering

properties, and likely play a role in maintaining the large pool of polymerization competent actin monomers.

The monomer sequestering activity observed for TgADF is consistent with its relatively high expression levels within the parasite, where it is found at almost equimolar levels with actin. If we assume that TgADF is the only binding partner of actin, with an affinity of 0.81 μM (Chapter 3), and TgADF and actin have cellular concentrations of 37 μM and 45 μM respectively (Chapter2), at equilibrium, ~82% of actin in the parasite is predicted to be bound to ADF. This indicates that the potential of ADF to sequester actin monomers is predicted to be quite large. However, TgADF is probably not the only binding partner for ATP-G-actin *in vivo*. Based on our knowledge from higher eukaryotic systems, profilin and CAP are also G-actin binding proteins with monomer sequestering activity, having typical binding affinities of 0.15 μM and 1.9 μM to ATP-G-actin, respectively (Didry *et al*., 1998; Mattila *et al*., 2004). *In vitro*, assays with heterologous actin predict that the parasite profilin and CAP proteins will also have roles in sequestering actin monomers (Kursula *et al*., 2008; Plattner *et al*., 2008; Hliscs *et al*., 2010). However, the cellular concentrations and affinities have not yet been determined for any of the parasite profilin or CAP proteins with parasite actin. Therefore, the likelihood of these interactions taking place *in vivo* is currently unknown. The cellular concentrations could be calculated by quantitative western blotting parasite lysate with specific antibodies against the protein of interest, while the affinity for actin could be calculated by measuring the dose-dependent effect of profilin and CAP on the rate of nucleotide exchange on G-actin, or by measuring the dose-dependent complex formation of the proteins with actin by native gel electrophoresis. The apparent affinity of

Cryptosporidium CAP for heterologous ATP-G-actin is calculated to be 0.78 μM (Hliscs *et al*., 2010), which is similar to the affinity of TgADF for parasite ATP-G-actin. If we assume that parasite profilin and CAP bind to parasite actin with affinities similar to their counterparts from other systems, and are as abundant in the parasite as TgADF is, then the largest proportion of ATP-G-actin in the parasite is predicted to be bound to profilin, based on its high affinity for ATP-G-actin, and ADF and CAP are expected to sequester the remaining actin monomers. The sequestering of actin monomers by these three proteins, could account for why polymerized actin is undetectable in apicomplexan parasites (Dobrowolski *et al*., 1997; Pinder *et al*., 1998; Shaw and Tilney, 1999), despite a cellular concentration of ~ 45 μM for actin in *T. gondii* (NS and LDS unpublished, and updated in Chapter 2). Defining the *in vitro* activities of ADF, profilin and CAP, and estimating their cellular concentrations, has the potential to significantly advance our understanding as to why the majority of actin is unpolymerized in apicomplexan parasites. Although we now have the data for TgADF, an important future direction will be to define these values for parasite profilin and CAP.

Analysis of the primary sequence of apicomplexan ADF proteins and homology modeling of TgADF (Chapter 3, Figure 1) and other apicomplexan ADF proteins (Schuler *et al.*, 2005; Xu *et al.*, 2008), reveals that the biochemical interactions observed between TgADF and actin are reflected in the molecular structure of apicomplexan ADFs. Although residues in the N-terminus and long α-helix that have been identified as important for G-actin binding are highly conserved in TgADF and other apicomplexan ADF proteins, sites involved in F-actin binding are absent or modified (Chapter 3). Specifically, charged residues at the C-terminus of AC proteins have been shown to be

important for F-actin binding (Lappalainen *et al.*, 1997; Ono *et al.*, 1999), and apicomplexan ADF proteins are noticeably truncated at the C-terminus (Chapter 3 and data not shown). Similarly, the F-loop, which refers to the β4 and β5 strands in the AC structure and is thought to directly interact with the filament (Lappalainen *et al.*, 1997; Pope *et al.*, 2000), is considerably shorter in the apicomplexan ADF proteins (Chapter 3)(Schuler *et al.*, 2005; Xu *et al.*, 2008), and likely only weakly interacts with filaments, if at all. Previous work has shown that mutation of the critical basic residue Lys-96 in the F-loop of human cofilin, which is essential for mediating interactions with the filament, can uncouple its severing and depolymerizing activities (Pope *et al.*, 2000). This mutation results in the loss of severing activity, and an increase in net filament disassembly activity. Apicomplexan ADF proteins have a natural variation at the corresponding residue (Gly-66 in TgADF), and correspondingly demonstrate weak interactions with actin filaments and strong net filament depolymerizing activity. Reversion to the conserved basic residue at this site had no effect on TgADF activity or its association with F-actin, consistent with the prediction that the shorter F-loop structure in TgADF does not significantly interact with filaments (Chapter 3). In contrast, addition of the C-terminal residues of the yeast cofilin protein to TgADF resulted in an increased association of TgADF with F-actin, and a corresponding decrease in its net filament disassembly activity (Chapter 3). This suggests that the presence of charged residues at the C-terminus of the protein can enhance the interaction of TgADF with actin filaments. Similarly, addition of the yeast cofilin F-loop sequence to TgADF to generate a longer F-loop structure, may also increase the association of TgADF with actin filaments, provided

the protein adopts a similar structure. The relevance of the F-loop structure for F-actin binding in apicomplexan ADF proteins will be examined in future work.

Overall, the molecular and structural features of TgADF and most apicomplexan ADF proteins are consistent with their role in functioning in a highly dynamic actin environment where most of the actin is unpolymerized, and filaments are polymerized only transiently. The absence of known F-actin binding sites in apicomplexan ADF proteins is reflected in less stable interactions with F-actin. Interestingly, the lack of F-actin binding sites appears to favor the binding of AC proteins to G-actin, as is seen for TgADF, or when corresponding mutations are introduced into more conventional AC proteins (Pope *et al.*, 2000). Thus, in the context of the G-actin rich cytoplasmic environment of the parasite, TgADF likely functions to maintain the high G-actin pool.

Despite lacking conserved F-actin binding sites, TgADF can still sever rabbit actin filaments (Chapter 3). Although higher concentrations of TgADF (1.5 μM) are required to detect similar levels of severing as yeast cofilin (0.3 μM), one of the most active severing proteins in the AC family (Andrianantoandro and Pollard, 2006), the concentration of TgADF required for severing is well within the levels of TgADF found in parasites (37 μM, Chapter 2). Interestingly, in non-motile parasites, TgADF is diffusely localized throughout the cytosol, whereas during gliding motility, TgADF is concentrated at the parasite periphery, where parasite actin filaments are also formed (Chapter 2). This switch in localization and local concentration could signify a concentration dependent regulation of TgADF activity. Consistent with this, low concentrations of TgADF are required to bind to G-actin and inhibit polymerization *in*

vitro, whereas an excess molar ratio of TgADF to F-actin was required to observe activity in the severing assay.

The relatively weak severing activity of TgADF on rabbit actin filaments compared to yeast cofilin, may also be functionally relevant for the disassembly of parasite actin filaments. Due to the less stable nature of parasite actin filaments, a lower rate of filament severing may be sufficient, and perhaps preferred, to cause filament turnover. Weak severing activity *in vitro* has previously been shown to be a biologically significant activity *in vivo*. For example, Unc60A, a *Caenorhabditis elegans* AC isoform with strong monomer sequestering and weak severing properties, is differentially expressed in early embryos and is essential for embryonic cytokinesis (Yamashiro *et al.*, 2005). Unc60B, a spliced variant that is normally expressed in the worm body-wall muscle, has strong severing activity and accelerates polymerization, but is unable to rescue Unc60A knockdown cells (Ono *et al.*, 2008). Instead, an Unc60B mutant that has reduced severing activity is able to partially rescue the Unc60A phenotype (Ono *et al.*, 2008). This suggests that the weak severing activity of Unc60A is functionally important in the cellular environment in which it is expressed, perhaps because strong filament severing activity may prevent transient filaments from being stable long enough to carry out their function. By analogy, the relatively weak severing activity of TgADF on rabbit actin filaments, may be optimal for the disassembly of *T. gondii* actin (TgACT) filaments, so that parasite actin filaments remain stable long enough to carry out their function. Alternatively, severing may not be a biologically significant activity of TgADF. To distinguish between these two possibilities, both *in vitro* and *in vivo* approaches could be utilized as discussed below.

In vitro, the severing activity of TgADF was demonstrated on rabbit actin filaments (Chapter 3). Although TgADF was shown to disassemble TgACT filaments in a bulk assay by sedimentation analysis (Chapter 3), and by direct observation using a static fluorescence microscopy assay with phalloidin stabilized TgACT filaments (Chapter 2), these assays did not address the kinetics or the mechanism of filament disassembly. The inability to generate long TgACT filaments *in vitro* without the presence of equimolar phalloidin stabilization had limited our ability to kinetically examine the activity of TgADF on TgACT filaments. However, recent data indicates that TgACT has two thresholds for interaction, and that spontaneous polymerization occurs above 5 μM TgACT (KMS and LDS, manuscript in preparation). Thus at high concentrations, TgACT is able to polymerize into longer filaments without phalloidin stabilization. Using this as a substrate, and by labeling filaments with a low level of fluorescently conjugated phalloidin, severing assays examined by TIRF microscopy could be used to address whether TgADF can efficiently disassemble TgACT filaments. Although the necessity for low levels of phalloidin for visualization of the filaments might dampen the observed kinetics, studies in Chapter 2 indicate that TgADF can act on phalloidin stabilized filaments, despite phalloidin being an antagonist for other AC proteins (Yonezawa *et al.*, 1988; Hayden *et al.*, 1993). Similarly, the low levels of phalloidin used for visualization of filaments, does not appear to have a significant effect on stabilizing TgACT filaments. Addition of TgADF should either increase the rate of TgACT filament disassembly, or have no effect compared to the addition of buffer alone or a control protein that only has sequestering activity. This experiment would address whether severing by TgADF can enhance TgACT filament disassembly *in vitro*.

Additionally, the significance of TgADF severing activity *in vivo*, could be addressed by conditionally expressing a TgADF mutant with strong severing activity in *T. gondii*, to determine if relatively weak filament severing is biologically important, or by adding back a severing null mutant in the TgADF conditional knockout background, to determine if severing activity is necessary *in vivo*. Resolving whether TgADF severing activity enhances TgACT filament turnover and if it is necessary *in vivo*, will be an important direction for future work.

Overall, my findings indicate that the main ways in which TgADF interacts with actin, is to sequester actin monomers and to sever actin filaments. Based on these interactions the following model is predicted (Figure 1). During non-motile stages of the parasite where the actin is almost exclusively unpolymerized, TgADF functions as a monomer sequestering protein, helping to maintain the high concentration of G-actin in the parasite. This is consistent with the diffuse colocalization of TgADF and actin throughout the cytoplasm in non-motile parasites. During gliding motility, when parasite filaments are transiently formed beneath the parasite plasma membrane and function in the translocation of surface adhesins, the severing activity of TgADF at the membrane promotes the turnover of parasite actin filaments, allowing for the high speed continuous movement that is observed during gliding. This switch in activity might be promoted by the high local concentration of TgADF at the parasite periphery during gliding and the proximity of TgADF to parasite filaments, or may require some modification to TgADF to reduce G-actin binding (discussed in the following section).

To test the role of TgADF *in vivo*, a parasite line was generated in which the expression of TgADF could be suppressed with the addition of anhydrous tetracycline,

effectively generating a conditional knockout of the protein (Chapter 4). These studies identified critical roles for TgADF in a number of steps in the *T. gondii* lytic cycle. Suppression of TgADF led to defects in parasite invasion, parasites were slow to egress from the vacuole, and parasites were unable to disperse from the vacuole after egress. The defects arose from the inability of parasites to undergo sustained gliding motility. Remarkably, parasites demonstrated a dramatic reduction in speed, moving at rates that were 82% slower than observed in wildtype cells. Interestingly a subpopulation of parasites engaged in a back and forth rocking motion that is not normally seen in wildtype parasites, which only engage in forward movement. This suggests that the turnover of old filaments may also provide directionality for parasite movement.

Since suppression of TgADF results in defects in motility, and this is the only stage at which parasite actin filaments are thought to form (Dobrowolski and Sibley, 1996; Shaw *et al.*, 2000), the phenotypes that are observed are likely due to decreased actin filament turnover during motility. Indeed the slow speed of parasite movement in the absence of TgADF, is reminiscent of what is observed with *Listeria* movement when actin-based motility is reconstituted *in vitro* using diluted platelet extracts (Carlier *et al.*, 1997). In the absence of exogenous ADF, bacterial movement is slow and long actin comet tails are observed. In contrast, increasing the concentration of ADF results in faster actin filament turnover, faster movement of bacteria, and shorter actin comet tails. By analogy, this could suggest that the defects in parasite motility observed with the suppression of TgADF are due to a dramatic reduction in filament turnover, which is required for continuous proccessive movement. This would be predicted to result in more stable actin filaments in the parasite, and a loss in the actin monomer pool over time.

Whether the motility defects are due to the presence of more stable filaments remains to be demonstrated, but could be examined by actin sedimentation analysis, by quantifying the amount of pelletable actin in wildtype parasites compared to parasites in which TgADF has been suppressed. If filaments are more stable when TgADF is suppressed, an increase in the pelletable actin is expected compared to the control. Alternatively, although more challenging due to the inherently short and transient nature of parasite actin filaments, but potentially more informative, direct visualization of actin filaments in the parasite may be possible by phalloidin staining gliding parasites and examining them by TIRF microscopy, or by examining the filament architecture underneath the parasite plasma membrane using freeze-fracture electron microscopy (Wetzel *et al.*, 2003; Sahoo *et al.*, 2006). Determining the effect of TgADF suppression on parasite actin *in vivo,* will be key to understanding how TgADF regulates actin dynamics and gliding motility in the parasite.

Although the *in vitro* data predicted an important role for TgADF in sequestering actin monomers during the non-motile parasite stages, no defects were observed at this stage with suppression of TgADF, under the conditions examined. This may be because TgADF likely functions in cooperation with other actin binding proteins such as profilin and CAP to maintain high G-actin concentrations in the parasite, and removal of just one of these proteins from the system may not have a significant effect. Similarly, no growth defect or abnormalities at the non-motile stage were reported for the *T. gondii* profilin conditional knockout (Plattner *et al.*, 2008). Additionally, it is possible that parasite actin filaments may be unlikely to form in the parasite cytoplasm in the absence of filament

nucleation and/or stabilization factors that may only be found in the inner membrane space (such as the formin1 protein).

If the model that TgADF serves two different functions depending on the motility state of the parasite is correct, there should be a switch regulating the shift between the two activities. In higher eukaryotes, AC protein activity is often negatively regulated by phosphorylation (Ono, 2007). I attempted to determine if TgADF is phosphorylated *in vivo* (Chapter 2), and although TgADF was post-translationally modified, the nature of the modifications was unable to be resolved, and there was no convincing evidence for phosphorylation. However, regulation by phosphorylation cannot be ruled out, and if TgADF is phosphoregulated, the phosphorylated form is likely to be transient. Therefore, to conclusively determine if phosphorylation has a role in regulating TgADF activity it would be necessary to examine this genetically using a nonphosphorylatable TgADF isoform. In many higher eukaryotes, AC proteins are kept inactivate by phosphorylation until activated by a signal transduction pathway for a specific activity such as cellular motility or cell division (Ono, 2007). In apicomplexan parasites, which lack a stable F-actin pool, ADF may be constitutively active in the non-motile stage, functioning to sequester actin monomers. In contrast, during gliding motility, when parasite actin filaments are polymerized, TgADF may regulate filament turnover by severing, and therefore the activity of TgADF on parasite actin filaments may also need to be regulated. Since other processes required for motility are initiated by signaling through the parasite calmodulin-like protein kinases (CDPKs), such as microneme exocytosis by CDPK1 (Lourido *et al.*, 2010), or *Plasmodium* ookinete motility by CDPK3 (Siden-Kiamos *et al.*, 2006), it is possible that phosphorylation might also be used to regulate a motility

specific activity of TgADF. Interestingly, a CDPK is found to regulate AC activity in plants (Allwood *et al.*, 2001). Phosphorylation on the serine 3 residue in TgADF (which is a conserved site of phosphorylation in animals, and is present in all of the apicomplexan ADF proteins), would decrease the affinity of TgADF for both F- and G-actin, thereby rendering it inactive. Recombinant TgADF that has the phosphomimetic S3E mutation demonstrates a loss of activity in *in vitro* sedimentation assays and filament disassembly assays (Chapter 2, Chapter 3). Inhibition of TgADF activity could subsequently be relieved by dephosphorylation by a specific phosphatase, allowing TgADF to interact with and possibly sever actin filaments, or bind to actin monomers.

Alternatively, interactions between TgADF and actin may be regulated by the presence of other actin binding proteins such as CAP and profilin, or other proteins that are specifically localized to the inner membrane space, that may have a higher affinity for ATP-G-actin than ADF. The binding affinities of the candidate proteins to *T. gondii* ATP-G-actin could be experimentally measured to identify which proteins have the highest affinity for actin and therefore which would be the most likely interaction partners *in vivo*. The ability of these proteins to compete with each other for actin binding *in vitro* could also be measured. Overall, determining how TgADF activity is regulated will be an important area of future research to understanding its function *in vivo*.

Summary

In summary, this body of work has provided a detailed analysis of how TgADF interacts with actin and found that TgADF sequesters actin monomers and severs actin filaments. These activities are reflected in the molecular features of TgADF and other parasite ADF

proteins. *In vivo*, TgADF activity is critical for *T. gondii* invasion, egress, and dispersal. A detailed analysis of motility after ADF suppression revealed that parasites were unable to undergo sustained motility, and moved with drastically reduced speeds. This study represents the first time where the *in vitro* activities of a parasite actin binding protein were investigated using the homologous apicomplexan actin substrate, lending credence that the interactions observed *in vitro* might bear relevance to the *in vivo* situation. The detailed analysis of parasite motility with ADF suppression suggests that ADF regulation of actin dynamics plays a critical role in regulating gliding motility. In addition to providing a scaffold to link transport of the surface adhesins to the myosin motor, the dynamic turnover of parasite actin may also play a role in translocating adhesins along the surface of the parasite. A better understanding of the function and regulation of the parasite actomyosin cytoskeleton in gliding motility could lead to targets for future therapeutics.

FUTURE DIRECTIONS

Identifying the basis of the motility defect with TgADF suppression

In vitro the main activities of TgADF are to sequester actin monomers and to sever actin filaments. Monomer sequestration is predicted to maintain a large G-actin pool, whereas severing activity would allow for the rapid turnover of filaments. Based on what is known from other motile systems and the *in vitro* data for TgADF, it would be predicted that the slow speed of parasite movement and the loss of sustained motility with

suppression of TgADF, is due to the reduction of filament turnover, and this is due to the lack of severing activity. However, this has not yet been demonstrated. One way to determine if severing is an important functional activity of TgADF would be to complement the TgADF conditional knockout with another protein that lacks severing activity and only has sequestering activity. The apicomplexan CAP proteins are an appropriate choice. CAP proteins in apicomplexan parasites only contain the C-terminal actin binding domain, lack the N-terminal signaling domain and do not form large macromolecular structures (Hliscs *et al.*, 2010). Work on *Cryptosporidium* CAP (CpCAP) reveals that it sequesters actin monomers and has an estimated affinity of 0.81 μM for heterologous actin (Hliscs *et al.*, 2010), which is similar to the affinity of TgADF for parasite actin (Chapter 3). CpCAP did not have any other interactions with actin *in vitro*, and based on its molecular structure, which is conserved in apicomplexan parasites and lacks the signaling domain and the WH2 domain found in higher eukaryotic CAP proteins, monomer sequestration is thought to be its only activity. This suggests that CpCAP may be able to functionally replace TgADF sequestering activity. To test this, CpCAP could be overexpressed in the *T. gondii* ADF conditional knockout using the tetracycline-on system, in which CpCAP would be expressed under the control of a tetracycline activated promoter (van Poppel *et al.*, 2006). If the motility defects that are observed when TgADF is suppressed are due to the loss of TgADF-mediated monomer sequestration, then overexpression of CpCAP may be able to rescue these defects. This could be assayed using gliding, invasion and egress assays, and CpCAP would be expected to restore wildtype gliding, invasion and egress. Alternatively, CpCAP might not be able to rescue the ADF suppression phenotype, which might suggest that the

monomer sequestration activity of TgADF is not responsible, or cannot fully account for the gliding defect, and that perhaps an alternative activity of TgADF, such as severing, is required for sustained gliding. Another possibility is that overexpression of CpCAP does not rescue the ADF suppression phenotype but results in a different phenotype. This could be due to some other activity or interaction of CpCAP *in vivo* that is currently unknown, and could be investigated further.

In order to more directly test if there is a biological role for TgADF severing, it would be necessary to uncouple the severing and sequestering activities of TgADF, since the two activities appear to be inversely related (Ono *et al.*, 1999; Pope *et al.*, 2000). This makes it challenging to determine if a phenotype is due to less severing activity, or stronger sequestering activity. Although TgADF lacks the known structural features that have been implicated in F-actin binding, it is still able to interact with filaments (Chapter 3). Using molecular modeling to dock TgADF onto the TgACT filament may reveal specific contact points between ADF and the filament. The importance of these sites could be validated *in vitro* by making point mutations in TgADF and testing the activity of these mutant TgADF proteins using the TIRF microscopy severing assay. If significant loss of activity is observed in the mutant TgADF compared to wildtype protein, the role of TgADF severing activity in gliding motility could be tested *in vivo*, by transfecting the TgADF mutants into the TgADF conditional knockout line. The ability of the mutants to rescue the suppression of wildtype TgADF should indicate whether TgADF severing activity is important *in vivo*.

Alternatively, to determine if the weak severing activity of TgADF is an important biological activity one could take advantage of the TgADF mutant, ADF-t, that

I generated (Chapter 3), which expresses the C-terminal F-actin binding domain of yeast cofilin. Studies indicate that AC proteins that have more stable interactions with F-actin, have increased severing activity (Ono *et al.*, 2001). I have previously shown *in vitro* using sedimentation analysis that the ADF-t mutant has a more stable association with rabbit actin filaments (Chapter 3). It is predicted that the ADF-t mutant would have increased severing activity compared to wildtype TgADF. This could be examined by comparing the activities of the ADF-t and wildtype TgADF recombinant proteins in severing assays by TIRF microscopy, using rabbit actin and *T. gondii* actin as substrates. To determine if, and to what extent sequestering activity of the ADF-t mutant is altered by the presence of the C-terminal residues, the effect of ADF-t on the steady-state polymerization of TgACT could be examined in comparison to wildtype TgADF. Although I have demonstrated that the presence of the C-terminal F-actin binding residues increased the association of ADF-t with rabbit actin filaments, it is unclear whether ADF-t demonstrates a more stable interaction with TgACT filaments. This would be assayed for by sedimentation analysis. After conducting the above preliminary characterization of the properties of the ADF-t mutant, and confirming that it demonstrates increased severing activity, ADF-t could be expressed in *T. gondii* to determine if the weak severing activity of TgADF is a biologically significant activity. If weak severing activity is a biologically important activity of wildtype TgADF, strong severing activity might be expected to be detrimental to the parasite. As ADF-t might have a dominant negative effect *in vivo*, its expression would be induced using the tetracycline-on system (van Poppel *et al.*, 2006), or the degradation domain system as described (Herm-Gotz *et al.*, 2007). In the degradation domain system the protein of

interest is expressed as a fusion protein with the destabilization domain ddFKBP targeting the protein for proteasomal degradation. In the presence of a synthetic ligand Shield-1, which reversibly binds to the destabilization domain, the protein is protected from the proteasomal machinery and the mutant isoform is expressed. In this way the function of the potentially dominant negative mutant could be assessed. The ADF-t mutant would be expressed in the background of the TgADF conditional knockout, to see if the ADF-t protein could rescue the gliding defects observed with suppression of TgADF, as assayed by gliding, invasion and egress assays. If weak severing is important for optimal disassembly of TgACT filaments, the phenotype with expression of the ADF-t mutant is expected to be more severe than with suppression of wildtype TgADF and parasites are expected to be unable to undergo gliding, invasion and egress, due to the inability of filaments to stay stable long enough to carry out their function. Alternatively if severing is not a relevant activity for TgADF function *in vivo*, then expression of the ADF-t mutant is expected to rescue TgADF suppression. Unfortunately, the ADF-t mutant may also be altered in sequestering activity, which could confound the interpretation, but any difference in ADF-t sequestering activity compared to wildtype TgADF sequestering activity could be measured *in vitro*, and taken into account when interpreting the *in vivo* results.

The C-terminus and the F-loop structure are the two sites in AC proteins that have been identified as being specifically required for binding to the actin filament. Apicomplexan ADF proteins have a short F-loop structure. Similar to how the addition of the yeast C-terminal F-actin binding site increased TgADF interaction with actin filaments, the importance of the F-loop in mediating TgADF binding to actin filaments

could be assessed by generating a TgADF F-loop mutant that has the sequence of the yeast cofilin F-loop, which should generate a TgADF protein with a longer F-loop structure. It would be important to verify that the protein is properly folded, which could be done by circular dichroism, and that the protein is still active, which could be assessed in sedimentation assays. To determine if a longer F-loop can increase and/or stabilize the interaction of TgADF with filaments, sedimentation analysis could be used to see if more of the F-loop mutant cosediments with actin filaments compared to wildtype TgADF. The F-loop mutant is expected to have similar properties as the ADF-t mutant expressing the C-terminal F-actin binding site, and could be analyzed in the same way. By expressing ADF mutants that have increased severing activity in the parasite and determining whether they are able to rescue the ADF suppression phenotype, or exacerbate the phenotype, should give some indication of whether severing by TgADF is an important activity *in vivo*.

In vivo, suppression of TgADF leads to defects in all processes that depend on parasite actin filaments and their turnover. Based on the *in vitro* activities of TgADF in monomer sequestering and filament severing, one would predict that defects from TgADF suppression *in vivo* arise from the presence of more stable filaments in the parasite. Specifically, an absence of sequestering activity might lead to an increase in the concentration of free G-actin (if it does not get sequestered by other G-actin binding proteins). The net result might be more filaments, and longer filaments. If lack of severing is the main cause for the motility defects, this would be predicted to result in longer-lived filaments in the parasites, due to a decreased rate of filament turnover, and perhaps longer filaments, as monomers would continue to be added on to existing

filaments. Examining the structure of actin filaments in ADF cKO gliding parasites might provide some insight into the observed defects. Phalloidin staining of actin filaments in gliding parasites could be attempted to determine the size and arrangement of filaments *in vivo* by fluorescence microscopy, although given the short length of parasite filaments, this may be challenging. Freeze-fracture electron microscopy of gliding parasites, although more technically challenging, could give more definitive results (Wetzel *et al.*, 2003; Sahoo *et al.*, 2006). If more filaments are present with ADF suppression, it may generate membrane footprints with a filament structure that resembles what is seen with jasplakinolide-treated parasites (Wetzel *et al.*, 2003; Sahoo *et al.*, 2006), that is more filaments with less of an orderly arrangement. To determine if actin filaments are longer with ADF suppression, the filament lengths could be quantitated from electron micrographs. Longer-lived filaments would be hard to distinguish, except that filaments might be captured at a higher frequency in the ADF suppressed parasites compared to wildtype parasites. Sedimentation analysis of actin from parasites in which TgADF has been suppressed compared to wildtype parasites may also allow for detection of changes in the parasite actin, but since it is a bulk assay, it would be less discriminating in terms of the mechanism.

Regulation of TgADF activity by phosphorylation

Although we now understand the potential ways in which TgADF can influence parasite actin dynamics, and that it is essential for controlling gliding motility, little is known about how the activities of TgADF are regulated. In many systems, AC activity is

negatively regulated by phosphorylation on a conserved N-terminal serine residue, and this decreases its affinity for both F- and G-actin (Bamburg, 1999). Phosphorylation has been shown to be a mechanism of AC regulation in vertebrate (Agnew *et al.*, 1995; Moriyama *et al.*, 1996; Meberg *et al.*, 1998), invertebrate (Ohashi *et al.*, 2000), and plant (Smertenko *et al.*, 1998) systems, and in Acanthamoeba, 30% of the AC pool is phosphorylated (Blanchoin *et al.*, 2000). However, phosphorylation does not regulate AC activity in yeast (Lappalainen *et al.*, 1997). Although we found that TgADF undergoes post-translational modifications *in vivo* (Chapter 2), and ^{32}P labeling of parasites suggests that TgADF may be phosphorylated *in vivo* (Chapter 2), we were unable to directly determine if TgADF is phosphorylated, or if another phosphorylated species was co-migrating with TgADF. Phosphorylation is unlikely to play a role in keeping ADF inactive during the non-motile stages, when it presumably functions to sequester G-actin. However, phosphorylation may play a role during motility to cycle TgADF on and off the filament. Such a mechanism has been seen in migrating cells where migratory signals often enhance AC phosphorylation and dephosphorylation, resulting in their cycling between inactive and active states (Ono, 2007). During gliding, the proximity of TgADF to the plasma membrane in the inner membrane space may also bring it into close proximity to signaling molecules that may respond to extracellular cues during motility. This could be tested experimentally using insight from other systems. For example, injection of the non-phosphorylatable S3A mutant into *Xenopus* oocytes revealed that although the *Xenopus* AC is dephosphorylated and recruited to the cleavage furrow at first cleavage, cycling between the active and inactive form is necessary for cleavage furrow progression (Abe *et al.*, 1996). In higher eukaryotes, phosphorylation is controlled

by the AC specific LIM kinase or TES kinase proteins (Ono, 2007), while in plants it is attributed to a calmodulin-like domain protein kinase (CDPK) (Allwood *et al.*, 2001), which are kinases found uniquely in plants and protists. Apicomplexan parasites express a diverse family of CDPKs (Billker *et al.*, 2009), some of which have recently been shown to be required for gliding motility (PfCDPK3 in *Plasmodium* ookinetes) (Siden-Kiamos *et al.*, 2006), or specifically to activate microneme exocytosis (TgCDPK1) (Lourido *et al.*, 2010), a process that is essential for gliding. It is possible that one of the CDPKs could be involved in signaling the recruitment or assembly of the actin machinery in the inner membrane space, and AC proteins have been shown to respond to such signals (Ono, 2007). The inactivation of AC proteins by dephosphorylation is mediated by the AC specific slingshot (Niwa *et al.*, 2002) or chronophin (Gohla *et al.*, 2005) phosphatases, the latter of which is found in apicomplexan parasites (Baum *et al.*, 2006; Schüler and Matuschewski, 2006).

To conclusively determine if phosphorylation has a role in regulating TgADF activity, the non-phosphorylatable S3C TgADF mutant could be expressed in the background of the TgADF conditional knockout that was generated in Chapter 4. The S3C mutant is a better choice for this experiment than the S3A mutant, which was found to have compromised activity in *in vitro* assays (Chapter 3). Initially, the mutant ADF isoform with an epitope tag could be directly transfected into the TgADF conditional knockout background, single-cell cloned, and tested to see if it can rescue the gliding, invasion and egress phenotypes observed when wildtype TgADF is suppressed. However, if phosphorylation of TgADF is an important regulatory mechanism, the expression of a constitutively active form of the protein could have a dominant-negative effect.

Therefore, a safer strategy would be to induce the expression of the S3C TgADF mutant in the background of the TgADF conditional knockout. This could be done using the tetracycline-on system (van Poppel *et al.*, 2006) in which the S3C TgADF would be expressed under the control of a tetracycline activated promoter. Alternatively, the degradation domain system could be used (Herm-Gotz *et al.*, 2007), as described in the previous section, to selectively stabilize a potentially dominant negative protein.

Experiments in Chapter 4 showed that suppression of ADF in the TgADF conditional knockout leads to parasites that are unable to undergo sustained gliding motility, and results in defects in parasite invasion and egress. Expression of the wildtype TgADF expressed in the same manner as the mutant, should complement the TgADF conditional knockout and restore normal gliding motility and related events. If phosphorylation plays a role in regulating TgADF activity, expression of the constitutively active S3C TgADF mutant will not rescue the TgADF conditional knockout and is expected to result in a more dramatic phenotype than just suppression of TgADF alone, resulting in no gliding, no invasion and no egress, due to the inability to form filaments. Alternatively if phosphorylation is not important, expression of the S3C mutant should behave similarly to the WT TgADF complement. A defect is not expected at the non-motile stage as actin is not thought to be required for parasite cell division or any intracellular processes (Dobrowolski and Sibley, 1996; Shaw *et al.*, 2000), and therefore TgADF is not expected to regulate actin activity at this stage, although this would need to be examined. If the S3C TgADF mutant is unable to significantly rescue the TgADF conditional knockout, it would suggest that phosphorylation may play a role in regulating TgADF activity.

The prime candidates for regulating TgADF phosphorylation directly or indirectly are the CDPK proteins (Billker *et al.*, 2009). Thus far the kinases mediating AC phosphorylation are fairly specific for their substrate, even in *in vitro* reactions (Bamburg, 1999). To identify candidate CDPK proteins that might phosphoregulate TgADF activity, the TgCDPKs could be expressed recombinantly, and screened for their ability to phosphorylate TgADF in *in vitro* phosphorylation reactions. Although the CDPKs might be permissive in their phosphorylation activity, it is worth performing this as an initial screen to narrow down the list of CDPKs of interest. Subsequently the candidate CDPKs would be conditionally expressed in *T. gondii* using the tetracycline-transactivator conditional knockout system (Meissner *et al.*, 2002). Briefly the CDPK would be cloned with an epitope tag under the control of an established tetracycline regulatable promoter (Meissner *et al.*, 2002) and transfected into the *T. gondii* strain expressing the tet-transactivator. Following selection, and isolation of stable clones, clones would be screened for their ability to regulate expression of the tagged CDPK allele. A conditional knockout would be generated by replacing the gene with a drug resistance marker, and inserting a downstream tandem YFPYFP tag as a negative selection marker to screen for non-homologous recombination events. Stable clones would be isolated and the absence of the endogenous CDPK gene confirmed by PCR analysis, and absence of the endogenous protein by western blot analysis. To determine if the CDPK regulates TgADF suppression one would screen for motility defects when the CDPK is suppressed, using gliding, invasion and egress assays. The CDPK of interest that phophoregulates TgADF should phenocopy the expression of the constitutively active S3C TgADF allele. That is, parasites are expected to be unable to glide, invade and

egress due to constitutively active TgADF, which is predicted to prevent the formation of parasite actin filaments. As the CDPKs are specific to plants and protists, if a CDPK is found to phosphorylate TgADF, a related CDPK in the other apicomplexan parasites is likely to control the same mechanism, and could represent a therapeutic target.

Regulation of TgADF activity by other actin binding proteins

Although the core actin binding proteins in apicomplexan parasites are thought to be identified, little is known about their specific properties and interactions with parasite actin, or how they function in regulating gliding motility. In order to have a better predictive capacity of the events occurring in the parasite cytoplasm, and to determine what the competing interactions for actin binding are, it would be useful to know the cellular concentrations of CAP and profilin in the parasite, and their binding affinities for both ADP- and ATP- *T. gondii* actin. To calculate the cellular concentration of a protein, for example CAP, recombinant protein could be expressed in *E. coli* and a specific antibody generated. This antibody could be used to determine the amount of CAP in parasite lysate by quantitative western blotting. To calculate the binding affinity of profilin for ADP- and ATP-G-actin, the nucleotide exchange rates of ADP- or ATP-G-actin in the presence of different concentrations of profilin could be measured. To do this the actin could be labeled with the 1,N^6-ethenoadenosine 5'-diphosphate (ε-ADP) or ε-ATP (Hawkins *et al.*, 1993), an ATP analogue which fluoresces when bound to actin, and the rate of nucleotide exchange measured as the loss in fluorescence over time when the nucleotide is displaced with unlabeled ATP. To measure the affinity of CAP for parasite

actin monomers, complex formation of CAP with ADP or ATP-G-actin could be measured by native gel electrophoresis and used to get an estimate of binding affinity. If the affinities for CAP and profilin are similar to their conventional homologues, one would expect that the majority of ATP-actin in the parasite is bound to profilin, and the remainder is buffered by ADF and CAP. This suggests that profilin would have the main function of transporting actin from the cytoplasm to the inner membrane space, where it could promote filament elongation on free barbed ends. However, if profilin has a moderate affinity for ATP-G-actin, then a sizeable proportion of ATP-G-actin may be bound to TgADF, and TgADF may function to transport the actin to the inner membrane space, raising the question of how ADF switches from sequestering monomers, to allowing polymers to form in the inner membrane space. AC proteins are known to respond to various signaling molecules and to redistribute to the leading edge during motility (Moon and Drubin, 1995; Ono, 2007). Relocalization to the parasite periphery is also observed for TgADF (Chapter 2). In higher eukaryotes the Rho GTPase family is the major regulator of the actin cytoskeleton mediating signals from the external environment (Etienne-Manneville and Hall, 2002; Jaffe and Hall, 2005). Rho GTPases are absent in apicomplexan parasites, suggesting that the signaling mechanism in apicomplexan parasites may be unique, possibly translated through one of the CDPK proteins.

Once in the inner membrane space, which one might predict to be a polymerizing environment due to the presence of a number of proteins that could potentially nucleate and/or stabilize parasite actin filaments (such as formin, aldolase, myosin), the question arises of how ADF is released from sequestering actin monomers. If profilin is present in the inner membrane space at reasonable concentrations, and has a much higher affinity

for ATP-G-actin, then it is likely to compete with ADF for binding to actin monomers. Thus, knowing the concentrations and affinities of various actin binding proteins with parasite actin would help to focus on the most relevant questions. Additionally, it would be useful to know the binding affinity of MIC2 to actin. Previous work (Travis Jewett thesis) suggests that MIC2 can bind directly to G-actin. This was shown *in vitro*, using MIC2 pulldowns of parasite lysate, and probing for the co-immunoprecipitation of aldolase and actin. A C-terminal glutamic acid residue (E755) in MIC2 was shown to be required for MIC2 association with actin, but not required for MIC2 binding to aldolase. If the affinity of MIC2 for ATP-G-actin is strong enough for MIC2 to compete for actin binding with other actin binding proteins such as ADF, profilin and CAP, it could suggest a mechanism for coupling the secretion of surface adhesins with assembly of the glideosome.

Regulation of TgADF activity by myosin

Although there are a small number of proteins that may compete with TgADF for binding to G-actin, apicomplexan parasites appear to lack F-actin binding proteins (with the exception of coronin), and specifically lack tropomyosin, a widely expressed actin binding protein that stabilizes filaments against AC proteins (Baum *et al.*, 2006; Schüler and Matuschewski, 2006). Interestingly, previous work has shown that myosin can compete with the binding of the AC proteins depactin and embryonic chicken skeletal muscle cofilin to actin (Mabuchi, 1982; Abe and Obinata, 1989). Both depactin and embryonic cofilin are similar to TgADF in that they inhibit actin polymerization and

appear to sequester actin monomers. For both proteins, myosin is able to overcome the inhibitory effects of these proteins on actin and promote actin polymerization. If myosin is able to compete with TgADF for actin binding, it might suggest that in the inner membrane space, interactions between TgADF and ATP-G-actin could be inhibited when the glideosome components come together, thus allowing for filament polymerization. To test if TgMyoA can compete with TgADF for actin binding, TgMyoA could be purified from *T. gondii* as previously described (Herm-Gotz *et al.*, 2002), and its effect on TgADF interactions with TgACT tested *in vitro* using a light scattering polymerization assay. Using different concentrations of TgMyoA, with a constant amount of TgADF and TgACT, one could determine if TgMyoA inhibits the interaction between TgADF and TgACT in a dose-dependent manner, and promotes actin polymerization. If TgMyoA exhibits this property, it would support a model where polymerization of TgACT filaments is promoted *in situ* as the glideosome assembles in the inner membrane space, and this model could be examined in more depth.

Determining the role of coronin in gliding motility

In recent years, most of the conserved actin binding proteins that are expressed in apicomplexan parasites have been generally characterized at the protein level, and in most cases exhibit the same general properties as the homologue in higher eukaryotes. Coronin is the last of the conserved actin binding proteins in apicomplexan parasites for which very little is known. In contrast to all the other actin binding proteins characterized thus far, coronin binds to the sides of the filament (Gandhi and Goode, 2008) and may

function to stabilize newly formed actin filaments in the inner membrane space. In order to study the role of coronin in the parasite, it would be most useful to generate a conditional knockout parasite line. This could be done in a similar manner as was done for TgADF in which a second copy of the gene is tagged and expressed under a tetracycline regulatable operator, and transformed into a parasite line expressing the tetracycline transactivator (Meissner *et al.*, 2002). After cloning and isolating a stable merodiploid line that expresses the tagged allele at similar levels to the endogenous gene, the endogenous gene could be replaced by a drug resistance gene by homologous recombination. Detailed analysis of the motility behavior of the coronin conditional knockout by live videomicroscopy would be instructive in determining the role of coronin in the parasite. If coronin helps to stabilize newly formed filaments in the inner membrane space against ADF activity, suppression of coronin would be predicted to result in actin filaments that have a faster turnover rate and are less stable. This could lead to faster but less coordinated movement of the parasite, and in the extreme case where filaments do not form, may result in non-motile parasites. Coronin would also be expressed recombinantly in *E. coli* to generate a specific antibody for localizing the protein within parasites, and for characterizing its activity in *in vitro* assays. Actin sedimentation assays with TgACT could be used to determine the F-actin binding activity of parasite coronin and to determine if it inhibits TgADF mediated depolymerization. Coronin protein could also be added to TgACT in *in vitro* filamentation assays and labeled with a small amount of fluorescently conjugated phalloidin to determine if it can stabilize actin filaments.

Despite our gains in understanding the activities of *T. gondii* ADF and how it interacts with *T. gondii* actin, and demonstrating its essential role in gliding motility, many questions still remain. Although TgADF only displays two main activities *in vitro*, namely sequestration of actin monomers and severing of actin filaments, and we can rationalize how these activities might function *in vivo* to regulate parasite actin dynamics, the actual mechanism by which TgADF functions *in vivo* is unknown. Based on our knowledge from higher eukaryotic systems, one might predict filament turnover to be the most important property of TgADF for regulating gliding motility. However since gliding motility and the properties of the apicomplexan parasite actin cytoskeleton are unique in comparison to the well-studied motility models, the mechanism of regulation of the parasite actin cytoskeleton may also be quite different. Questions that still remain to be answered include: What is the mechanism by which TgADF regulates gliding motility? How is ADF activity itself regulated? How are actin filaments stabilized in the parasite? These are all important questions that should be addressed in future work. In addition to potentially providing targets for future therapeutics, a better understanding of how the parasite actin cytoskeleton functions and is regulated may provide insight into how different biological systems have adapted the properties of actin to suit their unique lifestyle needs.

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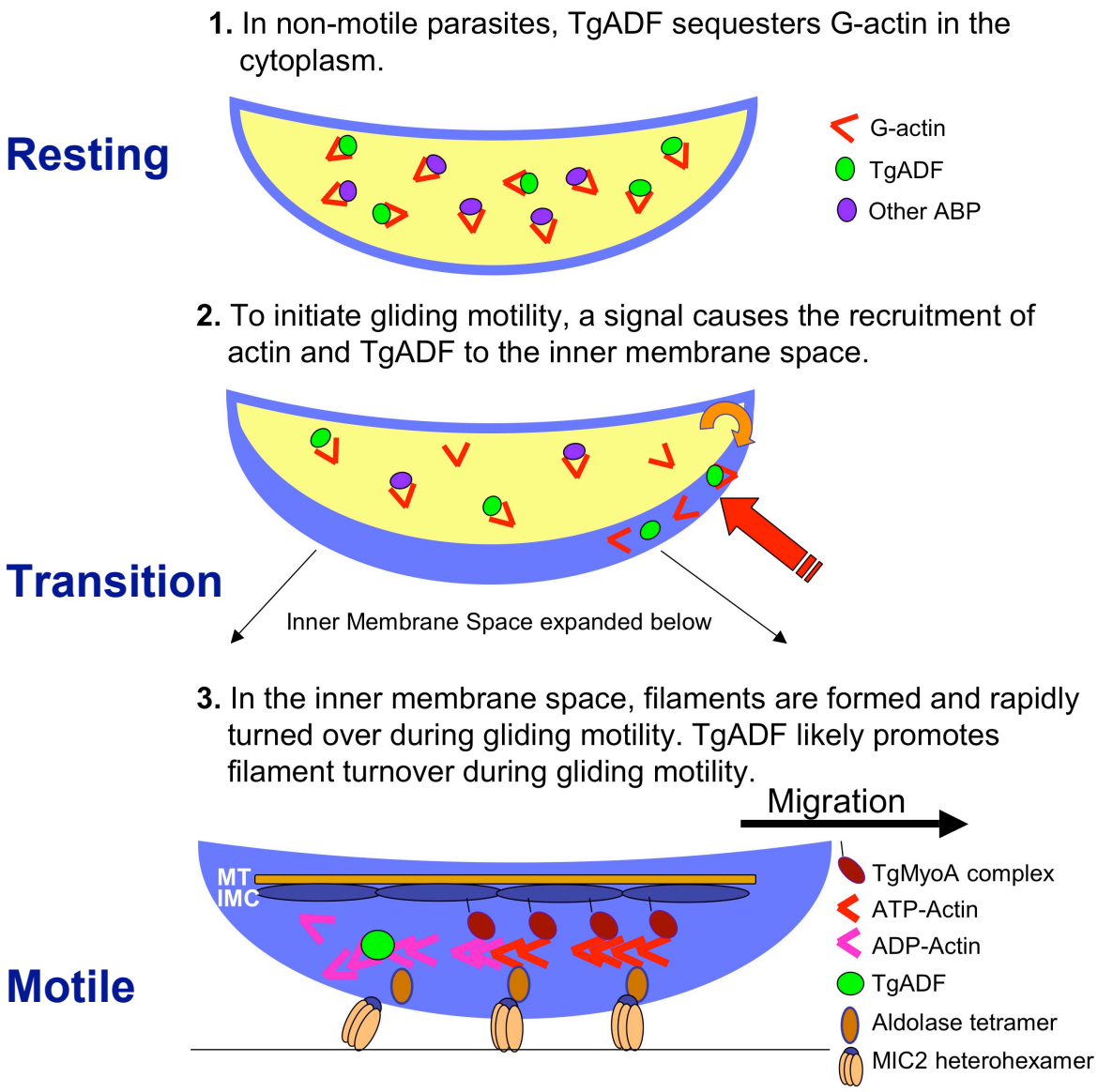

Figure 1. Model of how TgADF functions in regulating actin dynamics in *T. gondii*.