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Functional Comparison of *Toxoplasma* and Chicken Skeletal Muscle Actins in Motility Assays Using *Toxoplasma* Myosin

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April 2021

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Abstract

Toxoplasma gondii, an obligate intracellular apicomplexan parasite, utilizes gliding motility for active penetration and proactive egress from host cells. Gliding motility is made possible in apicomplexan parasites by use of the myosin motor complex, which is an actin-activated molecular motor. *Toxoplasma* actin is encoded by the single copy *ACT1* gene which is transcribed and translated into a single actin isoform and is inherently unstable in the filamentous form. In *T. gondii*, globular actin polymerizes to filamentous actin and is then driven rearward by the myosin motor during gliding motility. Prior research has shown differences between Apicomplexan and mammalian actin and myosin. The *in vitro* motility assay is used to study the *Toxoplasma* myosin motor complex, and chicken skeletal muscle actin is used in place of *Toxoplasma* actin. Here, recombinant *Toxoplasma* actin is expressed, purified, and polymerized in preparation for labeling and use in the *in vitro* motility assay. Confirmation of *Toxoplasma* actin expression was achieved by sequencing baculovirus encoding recombinant *Toxoplasma* actin and western blot analysis. Purification was achieved via nickel affinity chromatography, followed by cycles of polymerization and depolymerization. Preparing stabilized and labeled actin filaments allows for the comparison of the speeds *Toxoplasma* myosin moves *Toxoplasma* actin versus mammalian actin.

Author Summary

The Apicomplexa phylum, which includes *Toxoplasma gondii,* is responsible for a number of diseases (including toxoplasmosis, malaria, and cryptosporidiosis) and deaths worldwide. *T. gondii* requires the ability to invade and exit cells of the host to remain infectious as well as to replicate. This is all achieved by parasite motility – dependent on a complex of proteins, including actin and myosin, which are relatively well conserved within the Apicomplexa phylum. However, Apicomplexan actins differ in a number of key respects from mammalian actins. The motility assay utilized to study the parasite motor complex currently uses chicken skeletal muscle actin in place of *Toxoplasma* actin. We work here to express, purify, and stabilize *Toxoplasma* actin for use in the motility assay currently used to study the *T. gondii* motility complex, to enable a preliminary comparison of the interaction of chicken skeletal muscle actin versus *Toxoplasma* actin with recombinant *Toxoplasma* myosin motor complex.

Introduction

Toxoplasma gondii is capable of infecting nucleated cells of warm-blooded vertebrates, causing toxoplasmosis. Infections are typically caused by ingestion of contaminated food or water (1). *T. gondii*, an obligate intracellular apicomplexan parasite, utilizes gliding motility for active penetration and proactive egress from host tissues and cells (2-4). The study of Apicomplexan motility is important because motility is required to cause morbidity and mortality in hosts. Gliding motility is made possible in apicomplexan parasites by use of the myosin motor complex (Figure 1), which is an actin-activated molecular motor, situated within the cortical domain of the parasite. Rearward translocation of actin-associated cell surface adhesins is driven by the myosin motor complex anchored to an internal membrane complex, causing forward movement of the parasite (5, 6).

Apicomplexan myosin is composed of three domains: the force generating and actin-binding motor domain, the neck domain, and the C-terminal tail domain, which coordinates the light chains utilized in parasite motility (7). Myosin utilizes chemical energy from ATP hydrolysis to create mechanical energy. Motility in *T. gondii* involves Myosin A (TgMyoA), which is associated with two light chains, Myosin Light Chain 1 (TgMLC1) and Essential Light Chain 1 (TgELC1). When both TgMLC1 and TgELC1 are bound to the TgMyoA motor, the speed of actin movement is close to that of the motor complex isolated from parasites (8). To express a functional TgMyoA motor, a *T. gondii* myosin co-chaperone is required for proper folding of the TgMyoA heavy chain. This finding allowed for high yield expression of the TgMyoA motor complex and has allowed further detailed study of the *Toxoplasma* motor complex (8). It has also been shown that the TgMyoA heavy chain is one of 11 myosin heavy chains found in *T. gondii* and is a part of the TgMyoA motor located between the inner

membrane complex and plasma membrane in parasites (9). TgMyoA is distinguished from other myosin motors due to the presence of Gln, rather than an acidic residue or a phosphorylatable residue in a key actin binding surface loop that is otherwise highly conserved (10, 11).

Toxoplasma actin is encoded by the single copy *ACT1* gene which is transcribed and translated into a single actin isoform (5, 12). *Toxoplasma* actin, not host cell actin, has been shown to be essential for survival with gliding motility and host cell invasion both being compromised by lack of actin. Parasite egress is also not observed in the absence of parasite actin (4, 13, 14). Parasite actin is found primarily in the globular form (G-actin), and when filaments polymerize for motility, they are short and intrinsically unstable (15, 16). Filamentous actin (F-actin) consists of a right-handed helical spiral of two interwoven parallel strands formed by a self-polymerized head-to-tail dimer (17). The failure to generate cross-strand interactions that provide strong annealing may lead to the inherent instability of *T. gondii* ACT1 (TgACT1) (18). The abundance of F-actin has been shown to be rate limiting for motility, as the polymerization of actin filaments coordinates the initiation and directionality of movement (19).

In most eukaryotic cells, the sequence of actin is highly conserved, however apicomplexans encode more divergent actins. TgACT1 shows an 83% amino acid identity with mammalian muscle actin, and a 93.1% identity with *Plasmodium falciparum* actin (Figure 2a) (12, 15). Some amino acid differences can be seen at residues found to interact with the actin binding domain of myosin (Figure 2b, green boxes) (20). Additional amino acids interacting with the binding domain of myosin have also been identified (Figure 2b, orange boxes) (21). Apicomplexan parasite actins share divergent sequence features at the monomer surface with actins of close relatives, such as ciliates. These features have the potential to affect protein-protein interactions with actin-binding proteins or within the filaments. One such difference in *T.*

gondii is Arg277 (Figure 2b, red box), which is implicated in intermonomer salt bridge and hydrogen bond interactions. In TgACT1, this salt bridge is likely weakened due to an unfavorable Arg-Arg pairing (18). Another alteration in apicomplexan actins is seen within the hydrophobic plug. In *T. gondii,* according to the Holmes model, there would likely be additional destabilization between subunits across the filaments due to the alteration in the hydrophobic plug (Figure 2b, pink boxes). Experiments in which *Gly200* and *Lys270* in *Toxoplasma* actin have been substituted to the mammalian actin residue have shown *Toxoplasma* actin filaments that formed significantly longer filaments than the wild type *Toxoplasma* actin filaments (15, 18). The actin-binding proteins found within apicomplexans include actin depolymerizing factor, cyclase associated protein, profilin, and capping protein (22, 23).

Recombinant *Toxoplasma* actin (rTgACT1), produced in the baculovirus expression system, can be stabilized by the addition of equimolar levels of phalloidin to form long stable filaments that resemble conventional actin (15). Mapping of the phalloidin-binding domain in *Plasmodium falciparum* and *T. gondii* has shown six specific differences in residues contacting phalloidin in TgACT1 and PfACTII (actin in *P. falciparum*) versus mammalian muscle actin. These differences may cause the decreased binding efficiency of phalloidin to parasite actin filaments (15). Stabilization of *Toxoplasma* actin can also be achieved by the reversion of two key residues in *Toxoplasma* actin to match those of mammalian muscle actin (15). When expressed in parasites, these substitutions in TgACT1 modestly affected parasite cell division over the first 24-36 hours, and detrimentally affected gliding motility, which is supportive of conclusions drawn from Jasplakinolide (JAS) stabilization of actin (see below). Parasites expressing the stabilized actin moved more slowly, in circles with larger radii, and stalled while engaging in gliding motility (15).

JAS, a cyclopeptide, is useful for examining actin dynamics in live cells, as it is a membrane-permeable probe. JAS induces actin polymerization by binding to and stabilizing filaments, releasing monomeric actin from sequestering proteins, and decreasing the critical number of subunits necessary to induce polymerization (24, 25). When recombinant TgACT1 was treated with JAS for artificial stabilization, well defined TgACT1 filaments, with typical striations and slight helical twists, were formed *in vitro*. This led to cross linking between filaments, and an increase in overall length of filaments *in vitro*. However, JAS-treated TgACT1 filaments were still shorter than rabbit actin filaments (18). In parasites, JAS-treatment induced actin filament polymerization at specific sites rather than randomly, and increased TgACT1 lengths of filaments (18, 26). In addition, video microscopy of JAS-treated parasites showed they moved faster but with frequent direction reversals disrupting directed gliding (19).

The *Toxoplasma* motor complex has been assayed in *in vitro* motility assays to study TgMyoA function and demonstrated that actin moved at speeds similar to speeds of parasite motility on two dimensional surfaces and in three dimensional Matrigel (2, 27). The actin used in these *in vitro* motility assays however was rhodamine-phalloidin labeled chicken skeletal muscle actin, not actin from the parasite (8). A recent study demonstrated that *P. falciparum* MyoA moved *Plasmodium* actin filaments stabilized with JAS at the same speed as skeletal muscle actin (28).

As outlined above, prior research on the apicomplexan gliding motility motor complex has shown differences in actin, specifically TgACT1 is considerably divergent from mammalian actins which are typically highly conserved. Prior research has also demonstrated that human F-actin from skeletal and cardiac muscle, which differ at only four side chains, show differences in motility velocity with porcine β-myosin (29). It is unknown whether the *Toxoplasma* motor

complex will interact with and move *Toxoplasma* actin differently from chicken skeletal muscle actin in *in vitro* motility assays. While one study (28) has done some initial research with the *Plasmodium* motor complex and its ability to translocate *Plasmodium* versus skeletal muscle actin, further research is necessary.

With prior research demonstrating that there is the possibility for differences in motility and the way different F-actins interact with a given myosin, alongside the differences of *Toxoplasma* actin and myosin compared to mammalian actins and myosins, further study of TgMyoA function with the actin *T. gondii* utilizes for active penetration, proactive egress, and motility is necessary to determine whether there is a functional difference between *Toxoplasma* and chicken skeletal muscle actin in motility assays. In this study, we work to purify *Toxoplasma* actin from recombinant baculovirus encoding *Toxoplasma* actin, that has been polymerized and depolymerized, for use in the *in vitro* motility assay.

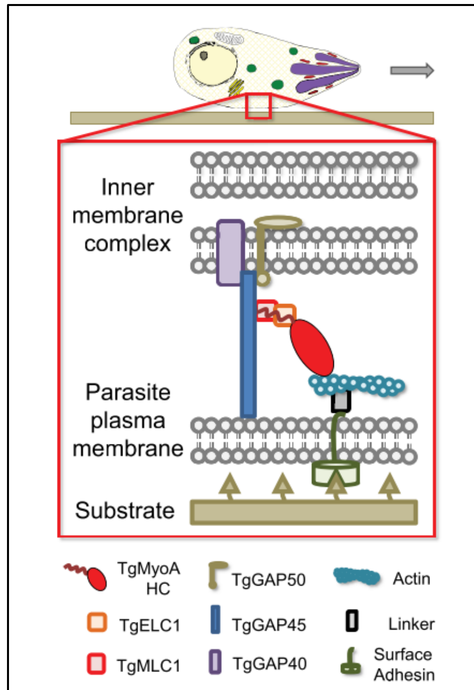

Figure 1. Schematic of *T. gondii* myosin motor complex. Previously published schematic of *T. gondii* myosin motor complex components (8). In the myosin motor complex, myosin associated with the inner membrane complex binds to actin associated with the parasite plasma membrane, and the actin is driven rearward. This rearward actin movement drive the parasite forward.

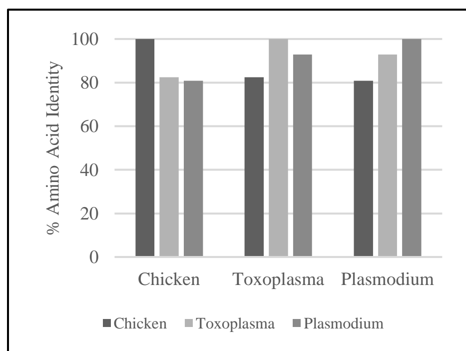

Figure 2a.

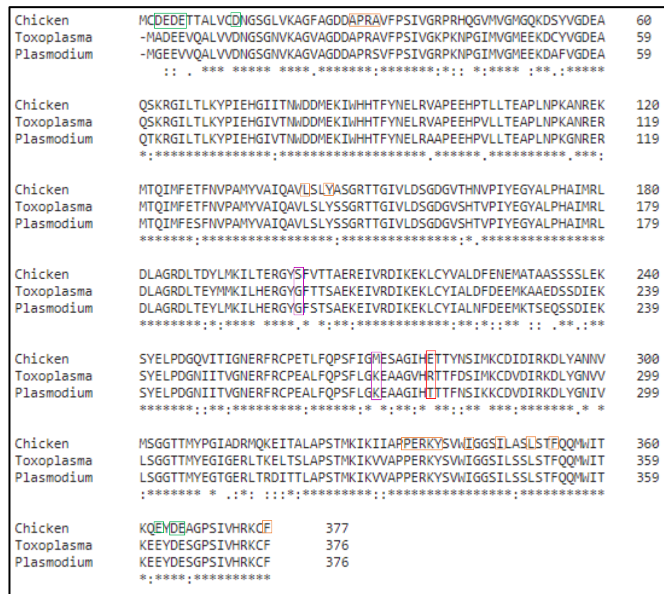

Figure 2b.

Figure 2. Actin Sequence Alignments. (a) Comparative amino acid identity percentage graphed based on (b) sequence alignment of Chicken skeletal muscle actin, *Toxoplasma* actin, and *Plasmodium* actin. Aligned using Clustal Omega multiple sequence aligner (30). Amino acids affecting actin filament stability highlighted in red (18) and purple (15). Amino acids interacting with the myosin actin binding domain highlighted in green (20) and orange (21).

Results

Successful expression of recombinant *Toxoplasma* actin in insect cells (Figure 3a), as well as confirming the baculovirus-encoded actin was both free of mutations (Figure 3b) and inserted in the vector as described (Figure 3c) (18), demonstrated the recombinant baculovirus and Sf9 insect cell system was an effective model for the expression of recombinant *Toxoplasma* actin.

Purification of *Toxoplasma* actin was achieved via nickel charged affinity chromatography (Figure 4a). Identification of the purified band as actin was confirmed by western blotting with an anti-actin antibody (Figure 4b). The His_6 tag was removed with thrombin to avoid any possible secondary effects on polymerization, depolymerization or any motility assay proteins. The slight decrease in molecular weight (to ~42kD) after His_6 removal is evident on the western blot (Figure 4b). To ensure that actin being fluorescently labeled for use in the *in vitro* motility assay was functional, the purified protein was taken through one cycle of polymerization/depolymerization (Figure 4c). From each 200 mL infection, approximately 2.9 mg of actin were recovered following nickel affinity purification, as determined by comparison of the actin band intensity to that of known amounts of low molecular weight standards on an Aqua Stained SDS gel (data not shown). With each step following affinity chromatography, 25-75% of the actin was lost.

TRITC-phalloidin binds to polymerized actin at the cleft between two monomers (31). Polymerized *Toxoplasma* actin bound by phalloidin was visualized (Figure 5a), however long filaments such as those formed by vertebrate actin were not formed (Figure 5b). Potential reasons for this are presented in the Discussion.

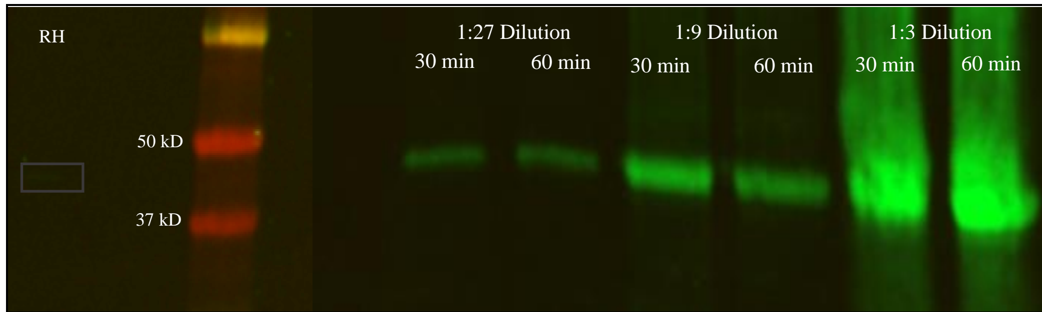

Figure 3a.

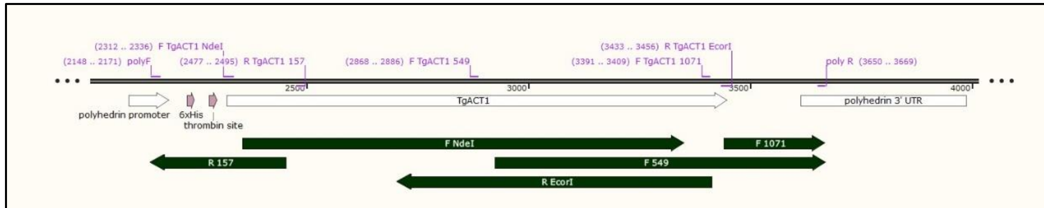

Figure 3b.

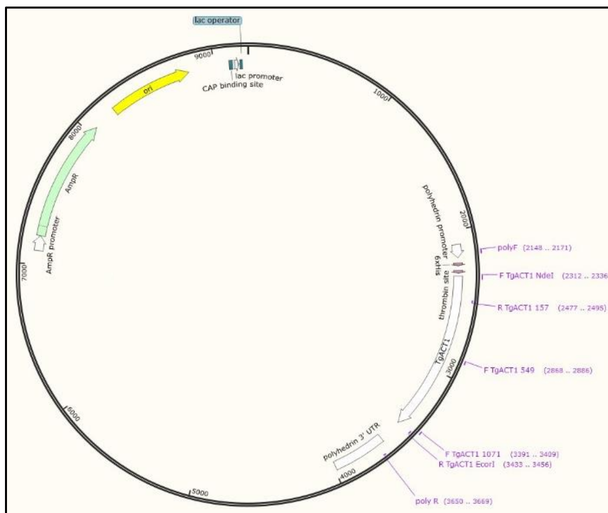

Figure 3c.

Figure 3. Verification of TgACT1 expression in Baculovirus system. (a) TgACT1 expression by Sf9 cells infected with baculovirus encoding recombinant TgACT1 was verified via 10% SDS-PAGE and western blot of insect cell extracts probed with rabbit anti-actin primary antibody. Different dilutions of the solubilized insect cell pellet (sonicated for either 30- or 60-minutes) were loaded, as indicated. RH strain parasites were loaded to provide an actin positive control (box). (b) Primer location and sequencing of recombinant TgACT1 in the baculovirus vector. Reference sequence (black bar) was constructed electronically via insertion of RH strain *T. gondii* cDNA sequence into pAcHLT-C vector backbone using NdeI and EcorI restriction enzymes. Purple text above reference sequence highlights primers used for sequencing. Green arrows indicate primers used, direction, and length of sequencing results. (c) Vector map of recombinant pAcHLT-C TgACT1 from the Sibley Lab (18), electronically constructed via insertion of RH strain *T. gondii* cDNA sequence into pAcHLT-C vector backbone using NdeI and EcorI restriction enzymes.

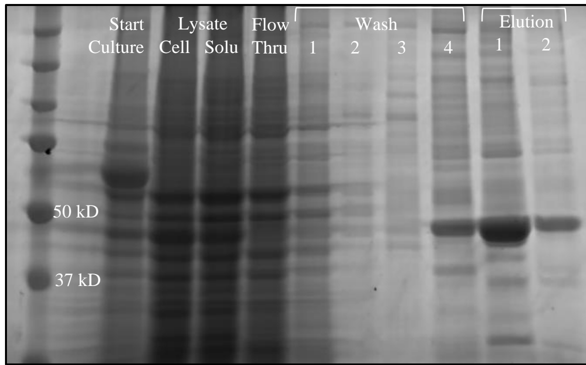

Figure 4a.

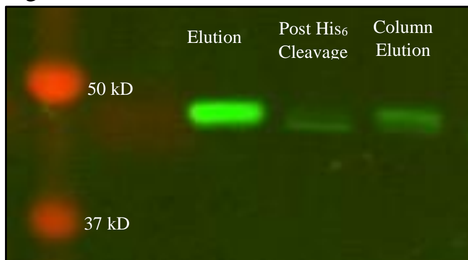

Figure 4b.

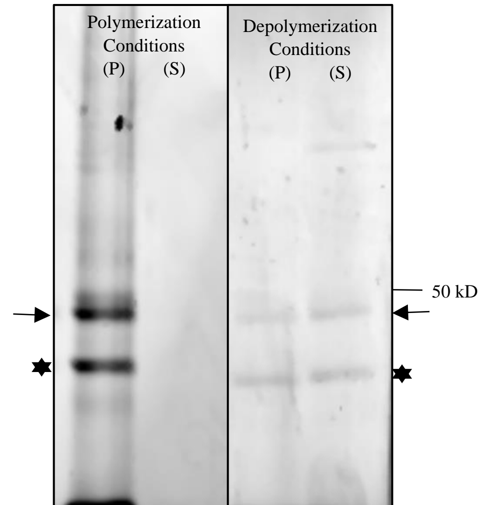

Figure 4c.

Figure 4. Purification and labeling of TgACT1. (a) His_6-TgACT1 (arrowhead) purified via nickel charged affinity resin was resolved on a 10% SDS-PAGE gel and visualized via Aqua Stain Protein Gel Stain. (b)Verification of TgACT1 presence after cleavage of His_6 from purified TgACT1. Samples were resolved via 10% SDS-PAGE and western blot probed with rabbit anti-actin primary antibody. (c) TgACT1 (arrow) was incubated with F-Actin Buffer salts (polymerization conditions) and then dialyzed against G-Actin Buffer (depolymerization conditions) and separated after each treatment into pellet (p) and supernatant (s) fractions by centrifugation at 300000*xg*. Samples resolved on a 10% SDS-PAGE gel and visualized via Aqua Stain Protein Gel Stain. Additional band (star) is an unknown contaminating protein that appears after thrombin cleavage.

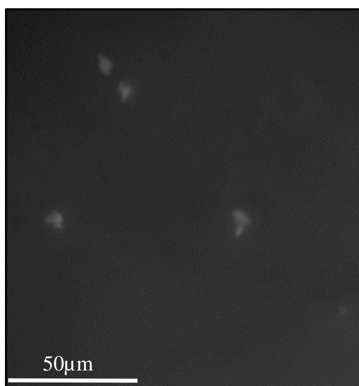

Figure 5a.

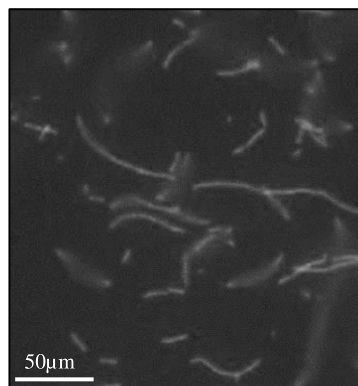

Figure 5b.

Figure 5. Visualization of polymerized actin. (a) TgACT1 stabilization and labeling was attempted with TRITC phalloidin; the phalloidin-treated samples were imaged by fluorescence imaging on a Nikon inverted epifluorescence microscope using NIS elements software. (b) Filamentous chicken skeletal muscle actin prepared by the Warshaw lab was imaged by epifluorescence imaging.

Discussion

Actin is a well-known protein with an important role in the motility of *Toxoplasma gondii* and other apicomplexan parasites. In addition, actin is utilized in motility assays studying many other aspects of the actin-myosin motor (8). We show here that it is possible to express stabilized recombinant *Toxoplasma* actin that has been through a cycle of polymerization and depolymerization.

The baculovirus expression vector system is widely utilized for recombinant protein production in insect cells due to the large quantities of protein it is able to produce (32). Expression of recombinant *Toxoplasma* actin still required several troubleshooting steps. First, it was necessary to ensure that the baculovirus expression system was producing recombinant actin and that no mutations were present in the actin sequence. This was accomplished by western blot and sequence analysis of TgACT1. Second, due to the viscosity of the insect cell pellet after infection with recombinant baculovirus and solubilization in sample buffer, the sample was unable to be run on the protein gel. To run the protein on the protein gel, and conduct western blot analysis, the cell pellet was water bath sonicated, and heavily diluted, producing clear results that confirmed the presence of *Toxoplasma* actin.

Successful purification of TgACT1 required additional troubleshooting. Previously published methods (15) to express and purify *Toxoplasma* actin merged with the manufacturer's protocol did not yield purified *Toxoplasma* actin. Rather it yielded a product with high amounts of contaminating protein that eluted earlier than expected off the column. To troubleshoot the purification wash and elution buffer volume, the protein was washed in greater volumes of wash buffers. However, it was determined that lower amount of imidazole in the wash buffers were necessary to remove contaminating proteins from the nickel affinity resin. An imidazole elution

gradient was conducted to determine the concentration at which, subjectively, the largest amount of contaminating protein was washed off the column, while losing the least amount of actin. Using data from these troubleshooting steps, *Toxoplasma* actin was purified from the nickel affinity column.

Polymerization kinetics and ion requirements of *Toxoplasma* actin and *Plasmodium* actin have been published previously; however, the findings vary between studies. It has been reported that the critical concentration of *Toxoplasma* actin, the concentration at which actin polymerization begins, was ~0.04µM (18). A later publication from the same group (33) reports, that upon reexamination, *Toxoplasma* actin polymerization is isodesmic, and therefore does not have a critical concentration. The previous findings are briefly addressed by stating the isodesmic conclusion is based off a larger range of actin polymerization concentrations while the previous publication only reported at low actin concentrations. Contrasting both results is a publication (34) which reports that the critical concentration of *Plasmodium* actin is ~4µM. These three reports describing the kinetics of apicomplexan actin filament formation highlight one of the difficulties in studying *Toxoplasma* actin, that there is no consensus on the dynamics of polymerization. In addition, the intrinsic instability of *Toxoplasma* actin has led to varied *g* forces used to sediment polymerized actin, with different groups achieving success at different *g* forces. Through many rounds of centrifuge speed trouble shooting, we were able to sediment polymerized actin (at 300000x*g*) and depolymerize previously sedimented actin, with the depolymerized actin remaining in the supernatant. The need to polymerize and depolymerize actin, prior to a final polymerization and stabilization step is supported by the intrinsic instability of *Toxoplasma* actin. Actin is a dynamic protein in the parasite, with actin remaining in filamentous form for extremely brief periods of time. Therefore, to best mimic parasite

conditions in the *in vitro* motility assay, the actin used needs to undergo similar dynamics prior to use in experimentation.

When stabilization and labeling of the polymerized actin was attempted with TRITC phalloidin, formation and/or stabilization of filaments did not occur. Previous research has shown at low actin concentrations, where phalloidin stabilization did not occur, *Toxoplasma* actin that had polymerized formed small clumps rather than filaments (33). The lack of stabilization into filaments may have been due to the length of time during which stabilization was attempted, or the concentration of phalloidin utilized. Further troubleshooting of polymerized *Toxoplasma* actin stabilization will be necessary prior to use in *in vitro* motility.

The *in vitro* motility assay is utilized frequently to study myosin function, small molecule inhibitors of myosin, and additional proteins associated with the myosin motor complex. Understanding whether the *in vitro* motility assay is capturing what is occurring in the parasite is important. In *in vitro* motility assays, if *Toxoplasma* myosin is shown to move *Toxoplasma* actin as the same speed as chicken skeletal muscle actin, the continued use of chicken skeletal muscle actin can be supported. However, if *Toxoplasma* actin moves at different speeds, it will be necessary to repeat previous experiments that have utilized the *in vitro* motility assay to elucidate whether differences in TgMyoAs interaction with *Toxoplasma* actin affect previously drawn conclusions. Additionally, utilization of the ATPase activity assay, to determine if the rate of ATP hydrolysis by *Toxoplasma* myosin varies in the presence of the different actins will further demonstrate if the continued use of chicken skeletal muscle actin can be supported when studying *T. gondii*'s motor complex. The continued research of the myosin motor complex as a target for pharmacological agents, as well as its importance for parasite motility justify the continued research of the associated proteins in the motor complex.

Materials and Methods

Cell Culture

Sf9 insect cells (Gibco Cell Culture, Gaithersburg, MD) were maintained in Serum Free Medium Complete (Gibco Cell Culture) containing 5% (v/v) heat inactivated fetal bovine serum (FBS), 1% (v/v) Penicillin/Streptomycin (10000U/mL / 10000ug/mL), and 0.05% (v/v) 50mg/mL Gentamicin Reagent Solution (Gibco Cell Culture) at 27°C.

Protein Staining and Western Blotting

Protein samples were boiled in SDS-PAGE sample buffer at 1X final concentration in the presence of β-mercaptoethanol for five minutes. Protein samples were resolved on a 10% SDS-PAGE gel and visualized by staining with Aqua Stain Protein Gel Stain (Bulldog Bio, Portsmouth, NH) or transferred to PVDF for western blot analysis. For western blots, membranes were blocked overnight at 4°C with Odyssey Blocking Buffer (LI-COR, Lincoln, NE) diluted 1:1 with TBS overnight at 4°C. Membranes were incubated for one hour with rabbit anti-actin primary antibody (Sibley Lab, St. Louis, MO) diluted 1:1000. Membranes were then washed three times for 15 minutes with TBST (0.15M NaCl, 0.02M Tris, pH 7.4, 1% v/v Tween-20) and incubated for one hour, in the dark, with goat-anti-rabbit secondary antibody (LI-COR) diluted 1:20000. Membranes were again washed three times for 15 minutes with TBST. Gel stains and western blots were imaged on LI-COR Odyssey CLx.

Toxoplasma Actin Expression and Sequence Verification

Recombinant TgACT1 was amplified by harvesting Sf9 cells infected with recombinant baculovirus containing TgACT1 (18) 7 days post infection. Infected Sf9 cells were then centrifuged (IEC Centra MP4R) at 800x*g* for 10 minutes. The pellet was resuspended in 1X SDS-PAGE sample buffer, boiled, water bath sonicated for 30 or 60 minutes, and resolved on a

10% SDS-PAGE gel followed by western blot.

Virus was recovered from the Sf9 infection supernatant by centrifugation at 20000x*g* for 15 minutes at 4°C in an ultracentrifuge (Optima TLX ultracentrifuge, Beckman TLA 100.3 rotor). The pellet was then resuspended in disruption buffer (1M Tris, pH 7.5, 0.1M EDTA, 10% SDS, proteinase K) and incubated overnight at 37°C in a water bath. Overnight solution was neutralized with neutralization solution and mini prepped (Promega, Madison, WI). gDNA was amplified by polymerase chain reaction with Phusion HF DNA Polymerase (New England Biolabs, Ipswich, MA) and polyhedrin forward and reverse primers (Table 1) in a Techne Prime Thermal Cycler (Staffordshire, UK). Amplified gDNA sequenced with TgACT1 R 157, TgACT1 R EcorI, TgACT1 F NdeI, TgACT1 F 549. and TgACT1 F 1071 primers (Table 1 & Figure 1b).

Primer Name	**Sequence**
Polyhedrin Forward	5'-TTTACTGTTTTCGTAACAGTTTTG-3'
Polyhedrin Reverse	5'-CAACAACGCACAGAATCTAG-3'
TgACT1 R 157	5'-GCTTCGTCACCGACGTAGC-3'
TgACT1 R EcorI	5'-GAATTCTTAGAAGCACTTGCGGTG-3'
TgACT1 F NdeI	5'-GCATATGTATGGCGGATGAAGAAGT-3'
TgACT1 F 549	5'-ACGCGACCTTACCGAGTAC-3'
TgACT1 F 1071	5'-ATCACCAAGGAGGAGTACG-3'

Table 1. Amplification and sequencing primers.

Toxoplasma Actin Expression and Purification

To express His_6-TgACT1 for Histidine affinity purification on a nickel affinity resin, $1*10^9$ Sf9 cells in 200mLs were infected with 3.25mLs 4X recombinant baculovirus containing TgACT1 for 48 hours. The cells were harvested and pelleted, at ~85% viability post infection, by centrifugation (RC5B Centrifuge, Sorvall GSA rotor) at 4000x*g* for 10 minutes at 4°C.

The cell pellet was resuspended in G-Actin Buffer (5mM Tris-Cl, pH 7.5, 0.2mM $CaCl_2$, 0.2mM ATP) containing 2% Triton X-100 and Protease inhibitor cocktail (Sigma p8340) and sonicated (Fisher Scientific, Waltham, MA) on ice at 20% amplitude for 5 seconds pulse on, 10 seconds

pulse off, for 4 cycles. Cell lysate was centrifuged at 3000x*g* (Sorvall SA600 rotor) for 15 minutes at 4°C. The post spin supernatant was transferred to Ni-NTA column (Invitrogen, Carlsbad, CA) and batch adsorbed for one hour at 4°C. The Ni-NTA column was washed with Native Wash Buffer (20mM $NaPO_4$, 500mM NaCl, pH 5.5, 0.5% Triton X-100), Native Wash Buffer with 0.5M KCl, Native Wash Buffer with 10mM Imidazole and Native Wash Buffer with 30mM Imidazole. His_6-TgACT1 was eluted with G-Actin Buffer containing 100mM Imidazole, followed by G-Actin Buffer containing 1M Imidazole.

His_6 Cleavage and TgACT1 Polymerization

His_6-TgACT1 eluted in G-Actin Buffer containing imidazole was dialyzed overnight at 4°C against G-Actin Buffer, and then incubated for 90 minutes with 1U of Thrombin per 10µg His_6-TgACT1 at room temperature. The actin was then transferred to the Ni-NTA column, batch adsorbed for one hour at 4°C, and the flow through containing TgACT1 collected.

F-Actin Buffer salts (100mM KCl, 2mM $MgCl_2$, 2mM ATP) were added to the flow through, incubated for 1 hour at room temperature followed by an overnight incubation at 4°C and centrifuged (Sorvall T885 rotor) at 300000x*g* in an ultracentrifuge (Sorvall Discovery 90 ultracentrifuge) at 4°C. The pellet was resuspended in F-Actin Buffer, dialyzed overnight at 4°C in G-Actin Buffer, and centrifuged at 300000x*g* at 4°C. F-Actin Buffer salts were again added to the supernatant and incubated for 1 hour at room temperature followed by an overnight incubation at 4°C. The overnight sample was incubated for 1 hour in the dark with TRITC phalloidin (Sigma-Aldrich, St. Louis, MO) at an equimolar concentration with the actin, and centrifuged at 200000x*g* at 4°C. Pelleted actin was resuspended in F-Actin Buffer.

Protein Quantification

Protein samples and low molecular weight standards (GE Healthcare, Buckinghamshire, UK) were run on Mini-PROTEAN TGX Stain-Free Gels (BIO-RAD, Hercules, CA), and visualized by staining with Aqua Stain Protein Gel Stain. Gel staining was imaged on LI-COR Odyssey CLx and analyzed with Image Studio Software.

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