KEENER, MARY ELIZABETH, M.S. Studies of the Actin Binding Activity of *Dictyostelium discoideum* Myosin II Heavy Chain Kinase A. (2008) Directed by Dr. Paul A. Steimle. 43 pp.

*Dictyostelium discoideum* is a primitive, eukaryotic organism that relies on myosin II and actin contraction for cytokinesis, migration, and other important cellular processes.  In order for contraction to occur, myosin II monomers must first assemble into bipolar filaments.  In *Dictyostelium*, bipolar filament assembly is negatively regulated by myosin heavy chain (MHC) phosphorylation.  Three kinases have been shown to catalyze MHC phosphorylation (MHK-A, -B, -C), driving myosin II disassembly.  Of the three, MHK-A is the most extensively studied.  Structurally, MHK-A is comprised of three functional domains:  an N-terminal coiled coil region (CC), a catalytic domain, and a WD repeat domain.  The CC domain has been shown, *in vitro*, to bind and bundle actin filaments and inhibit myosin II binding to F-actin.  The studies described in this thesis focus on two aims: 1) to understand the role of the CC domain in reorganizing myosin II and actin within the cell and 2) to determine if intramolecular interactions between the CC and WD repeat domains within the MHK-A molecule are involved in regulating MHK-A's activity.  To address the first aim, the triton cytoskeleton ghost isolation assay was conducted on Ax2, Ax2 + GFP-CC, Ax2 + GFP-C800A, MHK-A null, and MHK-A null + GFP-CC cell lines containing various truncations to determine the analyze the effects of over-expressing the CC domain on myosin II and F-actin organization within the cell.  Complementary localization studies were also conducted involving Ax2 wild-type cells and GFP-tagged cells over-expressing the

CC domain (GFP-CC) to visually determine myosin II and actin localization within the cell via confocal imaging. Results from the triton cytoskeleton studies indicate that over-expression of the CC domain in the Ax2 cells leads to an increase in cytoskeletal myosin II. MHK-A null and MHK-A null cells over-expressing the CC domain showed a significant increase in cytoskeletal myosin II in comparison to the Ax2 cells. However, the CC domain had no apparent effect in the absence of endogenous MHK-A as indicated by the relatively equal amounts of myosin II in both MHK-A null and MHK-null + GFP-CC pellet fractions. Ax2 + GFP-C800A cells exhibit essentially the same level of cytoskeletal myosin II compared to that of the Ax2 cells. Localization studies revealed increased amounts of cytoplasmically-localized myosin II aggregates in the Ax2 + GFP-CC cells in comparison to the Ax2 cells. Also, cells over-expressing the CC domain appear to have more cytoplasmic F-actin than the Ax2 cells. Furthermore, the actin appears to be more aggregated in the over-expressed CC cells. To address the second aim, purified CC and GST-tagged WD repeat domains were subjected to a GST "pull-down" assay and Western blot to determine if an interaction between the domains exists. Results from these experiments revealed no interaction between the WD repeat and CC domains, evident by the absence of CC in the GST-WD bead-associated fraction. This study shows that the CC domain plays an integral role in reorganizing the actin-myosin cytoskeleton and that the mechanisms regulating the full-length MHK-A do not appear to be determined by WD repeat and CC domain interactions.

STUDIES OF THE ACTIN BINDING ACTIVITY OF *DICTYOSTELIUM*

*DISCOIDEUM* MYOSIN II HEAVY CHAIN

KINASE A

By

Mary Elizabeth Keener

A Thesis Submitted to
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Approved by

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APPROVAL PAGE


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Date of Final Oral Examination


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# CHAPTER I

## INTRODUCTION

### *Dictyostelium discoideum*

*Dictyostelium discoideum* is a primitive, free-living eukaryotic organism that grows on dung and decaying vegetation where it feeds on bacteria residing there. It has a life cycle (Figure 1) characterized by a unicellular amoeboid stage and a multicellular "fruiting body" stage. *Dictyostelium* normally takes the form of individual amoebae when food is readily available. However, when food is scarce, the cells aggregate to form a multicellular mass called a pseudoplasmodium that resembles a slug. The slug form of *Dictyostelium* functions as a multicellular organism that has an anterior and a posterior and has the ability to migrate and respond to temperature and light. When conditions are favorable, slug migration stops and further development results in a fruiting body structure with a stalk supporting a capsule of spores made up of cellulose and glycoproteins. Disruption of the fruiting body capsule leads to the release of spores into the surrounding environment. If the area is nutrient rich, the spores will germinate, thus completing the developmental cycle with the release of the vegetative amoebae from within (Kessin, 2001).

*Dictyostelium* is a relatively simple, accessible, and informative organism used to study cellular processes common to most eukaryotic organisms, particularly

higher eukaryotes.  Furthermore, it is haploid for most of its life cycle, making it an organism with an easily manipulated genome.  Therefore, *Dictyostelium* studies have substantially beneficial implications in the areas of biomedical research, specifically in cell and developmental biology.  Some specific generalizations for eukaryotic cells have been developed from *Dictyostelium* studies.  For example, *Dictyostelium* studies have shown that conventional myosin is required for cytokinesis in eukaryotes (Mehta et al., 1999).  Furthermore, cloning and deletion of the cAMP receptors in *Dictyostelium* has shown that G-protein coupled receptors are essential for chemotaxis.  In leukocytes, for example, a family of twenty G-protein linked "chemokine" receptors function to mediate chemotaxis (Klein et al., 1988).  Therefore, *Dictyostelium* has advantages for studying processes such as cytokinesis, phagocytosis, chemotaxis, and other important cellular processes (Sasaki et al., 2002).

Dictyostelium has been a particularly useful model for understanding the roles of myosin II with regard to these cellular processes in a nonmuscle cell context.  Cellular biochemical, and genetic studies in *Dictyostelium* have revealed that myosin II plays a fundamental role in cell cytokinesis, cell migration, and multicellular development.  Twelve different types of myosin proteins are expressed in *Dictyostelium*, seven of which are isoforms of myosin I (a single-headed, unconventional myosin), and one of which is a double headed myosin protein known as myosin II (a conventional myosin).  Myosin II is a hexamer consisting of two regulatory light chains, two essential light chains, and two heavy chains.  Myosin II

contributes to vital cellular processes by driving the ATP-dependent contraction of actin filaments at specific locations within the cell. In order for myosin II to contract actin filaments, it must first assemble into bipolar filaments. In *Dictyostelium*, the formation of these filaments, and thus the contractile activity of myosin II, is negatively regulated by myosin heavy chain (MHC) phosphorylation (Figure 2) (De la Roche et al., 2002).

Actin and myosin II play fundamental roles in cellular processes such as cytokinesis, cell migration, and cellular development. In order for these processes to occur, myosin II monomers must first be assemble into bipolar filaments for ATP-dependent contraction of actin to occur. Filamentous actin (F-actin) is a cytoskeletal protein that localizes to the cortex of the cell and has cross-linking capabilities. Cross-linking refers to the linking of one or more actin polymer chains via covalent bonds, resulting in increased stability (Griffith and Pollard, 1982). The cross-linking ability of F-actin allows for pseudopod formation in response to chemotactic stimuli (Hall et al., 1989). Numerous studies have established the importance of actin-myosin II interactions in essential cellular processes. Cytokinesis studies have shown that myosin II is essential for contractile ring function and furrowing in suspension (De Lozanne and Spudich, 1987). MHC null cells are able to complete cell furrowing, however it is with frequent delays, inefficiency, and asymmetrical division (Neujahr et al., 1997). Cell migration studies using a non-phosphorylatable MHC mutant (3XALA) that exhibits severe over-assembly have shown that the

inability of the MHC to be phosphorylated leads to defects in chemotactic migration (Stites et al., 1998).

Three kinases have been shown to catalyze MHC phosphorylation, driving myosin II filament disassembly: Myosin Heavy Chain Kinase A (MHK-A), MHCK-B, and MHCK-C (De la Roche et al., 2002). MHK-A was the first kinase shown to catalyze myosin II filament disassembly both *in vitro* and *in vivo* and is the most extensively studied. Specifically, MHK-A phosphorylates three threonine residues on the carboxy-terminal domain of the *Dictyostelium* myosin II heavy chain to drive this disassembly (Cote and Bukiejko, 1987; Kolman et al., 1996). Previous studies have shown that when MHK-A is "knocked out", myosin II becomes over-assembled into functional bipolar filaments, leading to cell migration, cytokinesis, and development defects. MHK-A over-expression studies have shown that hyper-phosphorylation of the MHC via MHK-A leads to significant myosin II disassembly and defects in locomotion, cytokinesis, and cellular development. Structurally, MHK-A is composed of three functional domains: an N-terminal coiled coil region (CC), a catalytic domain, and a WD repeat domain (Figure 3) (Futey et al., 1995; Kolman and Egelhoff, 1997). Previous studies have shown that MHK-A activity is regulated by F-actin in that it can become activated in the presence of F-actin (Egelhoff et al., 2005). In turn, MHK-A can organize actin filaments into bundles, thereby inducing changes in the actin-myosin II cytoskeleton. This suggests that actin bundling, along with myosin II assembly and disassembly, is a physiologically relevant activity within the cell with regard to cellular processes. The CC domain of

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MHK-A has also been shown to bind and bundle actin filaments (Russ et al., 2006). Recent studies have also revealed that when the coiled-coil domain binds to F-actin, the interaction between myosin II and actin filaments is blocked (unpublished data). When this happens, myosin II filaments are unable to contract actin, thus prohibiting cellular processes that rely on the myosin-actin interaction. Thus, it is predicted that over-expression of the CC domain in cells will result in a myosin II-null phenotype, characterized by cytokinesis defects (unpublished data). These defects cause the cells to become large and multinucleated when grown in suspension culture.

Although MHK-A studies have revealed important characteristics of MHK-A, much is still not understood about the enzyme and its regulation in the cell. For example, it is not known if the actin-binding activity of MHK-A is a physiologically-relevant activity in the cross-linking and bundling of actin filaments in the cell. Moreover, it is unclear if intermolecular interactions within the MHK-A molecule are involved in regulating MHK-A's activity. The studies proposed for my thesis project will address both of these issues.

## **Thesis Research Aims**

The goal of this study was to see how *Dictyostelium* MHK-A regulates changes in the actin-myosin II cytoskeleton that are associated with cell shape changes which occur during cytokinesis and directed cell migration. My thesis research focused on understanding which properties of MHK-A (i.e. actin binding activity) might be targeted by signals that lead to very specific changes in

*Dictyostelium* cell shape.  More specifically, I examined the role that the CC domain

of MHK-A, which binds and cross-links actin filaments, plays in modulating the

reorganization of the cytoskeleton in *Dictyostelium* cells.  Based on previous *in vitro*

studies, I hypothesized that the CC domain of MHK-A binds and bundles actin

filaments *in vivo* leading to organizational changes in F-actin and myosin within the

cell.  I also hypothesized that MHK-A is regulated by an intermolecular interaction

resulting in a conformational change of MHK-A.  To test my hypotheses, I

conducted experiments that address the following questions:

1. **<u>Does the coiled-coil domain (CC) of MHCK A play a role in the</u>**

   **<u>reorganization of F-actin and myosin II in <i>Dictyostelium</i> cells?</u>**  To explore

   this possibility, I used wild-type and various mutated *Dictyostelium* cell lines

   These cell lines include:

   1. MHK-A null

   2. MHK-A null + GFP-CC

   3. Ax2 + GFP-CC

   4. Ax2 + GFP-C800A

   The MHK-A null cell line is characterized by a disrupted MHK-A gene and has

   been shown to exhibit significant over-assembly of myosin into the cytoskeleton

   (Kolman et al., 1996).  The MHK-A null + GFP-CC also has a disrupted MHK-

   A gene, but harbors a plasmid for over-expression of the CC domain with a GFP

   tag.  The Ax2 + GFP-CC cell line expresses endogenous MHK-A, but includes

the over-expressed CC domain of MHK-A. The Ax2 + GFP-C800A cell line is a mutation that includes the wild-type, full-length MHK-A but the cysteine residue 800 of MHK-A is converted to an alanine resulting in an inactive kinase (Steimle, et al 2002). All cell lines that included the exogenous CC domain are characterized by a GFP-tagged version of the domain (GFP-CC). The C800A cell line is also GFP-tagged (GFP-C800A). Since the CC domain of MHK-A has been shown to bind and bundle actin filaments *in vitro* (Russ et al., 2006), I predicted that F-actin in cells expressing elevated levels of the CC domain will have more of their F-actin organized into bundles than cells expressing wild-type levels of endogenous, full-length, MHK-A. Recent studies in the Steimle lab have also demonstrated that the CC domain of MHK-A binds to actin filaments in a manner that blocks the interaction of myosin II with F-actin (unpublished results), and thus the CC domain has the potential to disrupt myosin II-mediated contraction of actin filaments in the cell. The assembly state and organization of actin and myosin II were analyzed in wild-type and CC-over-expressing cell lines using the two methods listed below:

a. <u>Triton Cytoskeleton Assay</u>: This method allowed the comparison of actin and myosin II filament assembly levels among the cell lines described above maintained under different conditions (Steimle et al., 2001). The assay involved the lysis of cells with Triton X-100 detergent under conditions that preserve the filamentous state of F-actin. The cellular material was then fractionated via centrifugation to yield cytoskeleton-enriched pellets

containing F-actin and actin-associated proteins, including myosin II. Based on previous *in vitro* studies in which it was demonstrated that the CC domain binds to F-actin in a manner that results in actin filament bundling and that inhibits myosin II binding to F-actin (Russ et al., 2006), I predicted that cytoskeleton-enriched pellets from cells over-expressing the CC domain would have lower concentrations of myosin II than similar fractions prepared from wild-type cells.

b. Localization of F-actin and myosin II in fixed *Dictyostelium* cells: For these studies, Ax2 and Ax2+GFP-CC *Dictyostelium* cell lines were fixed under different conditions and then probed either with antibody that binds specifically to myosin II or with fluorescently-labeled phalloidin to visualize actin filaments. Based on the results from previous *in vitro* experiments (Russ et al., 2006), I predicted that the F-actin in CC-over-expressing cells would be organized into aggregates localized throughout the cell. By contrast, numerous other studies have shown that F-actin is localized mainly to the cortex of wild-type cells with very few detectable aggregates (Lee et al., 1998). Because myosin II co-localizes with F-actin, myosin II is also localized to the cortex of wild-type cells. If the CC domain inhibits myosin II binding to actin filaments *in vivo*, then over-expression of the CC domain should reduce the amount of cortically-localized myosin II.

**2. <u>Do the WD-repeat and CC domains of MHCK A interact with each other?</u>**

This is an important question since an intermolecular interaction between these two domains is a potential mechanism for regulating the various activities of MHCK A. While there are no reports of WD-repeat and CC domains interacting with each other in any biological system, the locations of these domains (opposite ends of MHK-A) indicate that they may interact to induce changes in MHK-A structure and activity. This question was addressed by performing experiments in which a purified GST-fusion protein containing the WD-repeat domain (GST-WD) of MHK-A was incubated with purified CC domain protein (lacking a GST tag). An interaction between these two proteins was determined by "pulling down" the GST-WD protein with glutathione agarose beads and then analyzing the bead-associated and soluble fractions (via SDS-PAGE) for the presence of CC protein. I predicted that an interaction would exist, indicated by the presence of both the GST-WD and CC proteins in the bead-associated fraction.

# CHAPTER II

## MATERIALS AND METHODS

### *Dictyostelium* Maintenance

*Dictyostelium* cells (Ax2 wild-type strain) were cultured on 10 cm Petri dishes containing HL5 medium containing penicillin and streptomycin, unless otherwise indicated (Sussman, 1987). Cells containing plasmids were supplemented with the antibiotic G418 (10 μg/mol) to allow growth of only cells with plasmids encoding for G418 resistance.

### Triton Cytoskeletal Ghost Isolation

This assay allows for the quantification of the amount of myosin II and actin in the "Triton-resistant cytoskeleton" and is used to estimate the level of filamentous actin (F-actin) and assembled myosin II in the cell (Steimle et al., 2001). Cells from plates were collected and counted ($5 \times 10^6$ cells). Cells were then washed by centrifugation (1500 rpm for seven minutes) and resuspended in 1 ml of Starvation Buffer (20 mM MES pH 6.8, 0.2 mM $CaCl_2$, and 2 mM $MgCl_2$) for a total of three washes. After the final wash, the cells were resuspended in 1 ml of Starvation Buffer and 300 μl of each cell suspension were transferred into a 1.5 ml microfuge tube ("pellet" tubes). The cells were centrifuged again (1500 rpm for seven minutes). While the cells were centrifuging, 700 μl of acetone were transferred to 1.5 ml

microfuge tubes (the corresponding "supernatant tubes") and placed on ice. Post-centrifugation, the supernatant was removed from each pellet tube and the cells were resuspended in 150 μl of Buffer A (0.1 M MES pH 6.8, 2.5 mM EGTA, 5 mM MgCl₂, 0.5 mM ATP). Once the cells were resuspended, 150 μl of Buffer B (87.5 % Buffer A, 2.5 % Triton X-100, 1x Protease Inhibitor Cocktail, Roche Applied Science) were then added and the tubes vortexed for five seconds and centrifuged at 14,000 rpm for one minute. The supernatant from each tube was removed and placed into the corresponding supernatant microfuge tube containing acetone. The supernatant tubes were left on ice for 15 minutes allowing the acetone to precipitate the supernatant proteins. During that time, 25 μl of pre-heated (95° C) 5x sample buffer (95% 2x SB stock and 5 % ß-mercaptoethanol) were added to the pellet tubes, pellets were resuspended, heated at 95°C for five minutes, and placed on ice until ready to load onto the gel. After fifteen minutes had passed, the supernatant tubes were centrifuged at 14,000 rpm for fifteen minutes. The supernatant was discarded and the tubes carefully air-dried to remove any residual acetone. Once dried, 25 μl of pre-heated 5x sample buffer were added to the tubes, pellets were resuspended, and placed on ice until ready to load onto the gel. Samples (entire amounts) were loaded onto a 10% acrylamide gel along with 10 μl of an unstained protein standard. The gel was run at 150 Volts in SDS-PAGE running buffer, stained with Imperial Protein Stain (Pierce, Rockford, IL), and destained with distilled water.

## GST Fusion Protein Pull-down Assay

This assay tests for the interaction between CC and the WD-repeat domains of MHK-A using three binding reactions: (1) GST-WD + CC, (2) GST + CC, and (3) CC alone. The reactions (50 μl) were first prepared in 1.5 ml microfuge tubes as follows:

1. 0.3 μM CC
   1.5 μM GST-WD
   1x PBS (Phosphate Buffered Saline)
   37 μl dH$_2$O

2. 0.3 μM CC
   4 μl diluted GST (1 μl GST in 9 μl dH$_2$O)
   1x PBS
   37 μl dH$_2$O

3. 0.3 μM CC
   1x PBS
   41 μl dH$_2$O

Once the 50 μl reaction mixes were prepared, the glutathione-agarose beads (Novagen) were prepared by pipetting 160 μl of the 50% bead slurry into a 1.5 ml microfuge tube, adding 1 ml 1x PBS (10mM Na-Phosphate and 100mM NaCl), and spinning the mixture at 2,000 rpm for three minutes. The supernatant was carefully removed and the wash steps were repeated three times beginning with the addition of 1 ml 1x PBS to the pellet. After the third wash, the beads were resuspended in 160 μl of 1x PBS and 60 μl of the bead mixture were transferred to three microfuge tubes. The microfuge tubes were centrifuged at 2,000 rpm for three minutes and the supernatant carefully discarded. The previously prepared 50 μl binding reactions were then transferred to the tubes containing the beads. The reaction/bead slurry was then incubated for thirty minutes at room temperature with rotation. Once incubation was complete, the tubes were centrifuged at 2,000 rpm for three minutes. The supernatant was carefully collected and transferred to fresh microfuge tubes and

placed on ice.  The remaining pellets were washed twice by adding 1 ml of 1x PBS,

centrifuging at 2,000 rpm for three minutes, and carefully removing and discarding

the supernatant.  After the supernatant was discarded, 50 μl of 1x PBS were added to

the pellets.  Pre-heated (95 °C) SDS-PAGE sample buffer was added to both

supernatant and pellet tubes in the amount of 15 μl.  The localization of CC protein

in the pellet and supernatant fractions were then analyzed by SDS-PAGE/ Coomassie

Blue Staining and Western Blotting probing with anti-MHK-A antibody.


**<u>Fusion Protein Purification</u>**

This procedure allows for the purification of GST-WD from bacterial

cultures for use in the GST-binding assays and was performed essentially as

described by Steimle et al. (2001).  A colony of bacterial cells expressing GST-WD

was first placed in 10 ml of Luria Broth (LB) media containing 100μg/ml of filter-

sterilized ampicillin and grown overnight at 37°C with shaking (200 rpm).  The

following day, the 10 ml bacterial culture was transferred to 250 ml of LB media

containing 100μg/ml of filter-sterilized ampicillin.  An initial reading of bacterial cell

density was taken with a spectrophotometer.  The bacterial culture was then

incubated at 37°C with shaking and periodic spectrophotometric measurements taken

until cell growth was sufficient (OD value between 0.600-1.00 nanometers).  After

the final spectrophotometer reading the culture was cooled to room temperature and

250 μl of Isopropyl ß-D-1-thiogalactopyranoside (0.24 M IPTG) were transferred to

the flask.  The culture was then incubated at room temperature with shaking for three

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placed on ice.  The remaining pellets were washed twice by adding 1 ml of 1x PBS,

centrifuging at 2,000 rpm for three minutes, and carefully removing and discarding

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250 μl of Isopropyl ß-D-1-thiogalactopyranoside (0.24 M IPTG) were transferred to

the flask.  The culture was then incubated at room temperature with shaking for three

hours to overnight. After incubation, the cells were centrifuged at 6,000 rpm for 15 minutes. The supernatant was discarded and the cell pellet was placed in a freezer at -20°C for 30 minutes. The cells were then completely thawed on ice followed by the addition of 5 ml of 1x PBS and 0.025g of lysozyme (MP Biomedicals, cat. #100831). The cells were placed on ice for 10 minutes, and 20 µl of 0.5M ethylenediaminetetraacetic acid (EDTA), 50 µl of phenylmethylsulphonyl fluoride (PMSF, 20mg/ml in isopropanol), and 500 µl of 10x protease inhibitor cocktail (PIC) were added. The cells were incubated on ice for 10 minutes and then sonicated four times for 30 seconds each with 30 second intervals in between. Once the cells were transferred to 1.5 ml tubes, the tubes were centrifuged at 16,400 rpm for 30 minutes at 4°C. All of the supernatant was collected and transferred to 250 µl of glutathione-agarose beads (Novagen, cat. #70541) that had been washed four times with 10 ml of 1x PBS, and the entire bead slurry was incubated overnight at 4°C with rotation. The following day the bead slurry was transferred to a column and the unbound material was discarded. The remaining beads were resuspended in 1 ml 1x PBS and transferred to a 15 ml conical tube and washed eight times with 10 ml of 1x PBS. The slurry was then transferred to a microfuge tube and centrifuged at 3,000 rpm for five minutes. The supernatant was discarded and the pellet was placed on ice until the next step. To elute the GST-Fusion protein from the beads, GST-fusion Protein Purification Elution Buffer was then added (10x GST-Elution Buffer, 10x PIC, 0.2M PMSF, 0.5M EDTA) to the pellet and the entire mixture was incubated for one hour to overnight with rotation at 4°C. After incubation the slurry was transferred to a

mini column and centrifuged to collect the eluate.  The yield and purity of the GST-Fusion protein was assessed by analyzing an aliquot of the eluate via SDS-Page and Coomassie Blue Staining.  Once purification was confirmed, the remaining eluate was transferred to a dialysis cassette (Slide-A-Lyzer, cat. #66370) and placed in dialysis buffer (2L) overnight (10mM Tris pH 7.5, 10% Glycerol, 50mM NaCl, 0.5 mM EDTA, and 0.5 mM dithiothreitol (DTT)) with a stir bar at 4°C.  The GST-WD protein was then collected from the membrane and quantified via SDS-Page and Coomassie Blue Staining.  Purification of the CC domain of MHK-A without a GST tag was performed according to the protocol previously described (Russ et al., 2006).

**Labeling of Cells for Visualization of F-Actin and Myosin II**

These procedures permit the visual localization of F-actin and myosin II using confocal microscopy and various staining techniques.  For F-actin visualization, Ax2 and Ax2 + GFP-CC cells were collected and counted and $10^5$ cells of each cell line are transferred to separate slides.  The slides were prepared by drawing a ~1 cm circle using a Pap Pen in the center of each slide.  Once the cells were transferred to the prepped slides, they were allowed to adhere for 15 minutes followed by fixation via paraformaldehyde (4% in starvation buffer) for 10 minutes at room temperature.  Cell membranes were then permeabilized by washing in 0.02% Triton X-100 in 1x PBS for 2 minutes.  Cells were then washed 3 times in 1x PBS (100 μl per slide) for 2 minutes each.  Following the washes, cells were incubated with Texas-Red Phalloidin (2 μM in 1x PBS) for one hour to overnight at room

temperature.  After staining, the cells were washed 3 times in 1x PBS (100 μl per slide) for 2 minutes each.  Cells were then viewed under the confocal microscope to visually detect the presence and localization of F-actin.

To visualize Myosin II localization, Ax2 and GFP Coil cells were collected and counted as previously described and allowed to adhere to prepared slides.  Once the cells adhered to the slides, the slides were placed in beakers containing 100% methanol (10°C) for 5 minutes to allow for fixation.  Slides were removed and placed in a beaker containing 1x PBS and allowed to sit for 2 minutes.  This step was repeated once more in another beaker with fresh 1x PBS.  After washing, the slides were transferred to a moist chamber and 100 μl of blocking solution (3% Bovine Serum Albumin in 1x PBS) were added to the slides.  After 1 hour, the blocking solution was removed and replaced with 100 μl of anti-Myosin primary antibody (1:5000 in 3% BSA).  Following the 1 hour incubation period, the antibody was removed and the cells were washed 2 times with 1x PBS for 2 minutes.  Cells were then probed with Alexi-Fluor 546 goat anti-rabbit secondary antibody (1:1000 in 3% BSA) and left to incubate for 1 hour.  After incubation, the cells were washed 2 times in 1x PBS for 2 minutes.  Cells were then viewed under the confocal microscope to visually detect the presence and localization of myosin II.

## Cell Lines

The MHK-A null, MHK-A null + GFP-CC, and Ax2 + GFP-C800A cell lines were generated according to the protocols previously described (Steimle et al., 2002). The Ax2 + GFP-CC cell line was generated by electroporating the plasmid for over-expression of GFP-CC (Steimle et al., 2002) into Ax2 cells (Watts and Ashworth, 1970) expressing endogenous MHK-A. To electroporate, Ax2 cells were collected and counted ($5 \times 10^6$). Cells were centrifuged at 1500 rpm for 5 minutes, followed by two washes with ice-cold H-50 buffer (5 ml per wash). The pellets were then resuspended in 100 μl of H-50 buffer. PA-Coil-GFP (5 μl) was added to the cells after resuspension. The cell suspension (100 μl) was then transferred to a 0.1 cm electroporation cuvette, followed by electroporation at 0.85 kV/25 mF (time constant ~0.6 msec). The cells were electroporated twice without interval and incubated on ice for 5 minutes. After incubation, 1 ml of HL-5 media was added to the cuvette and the entire cell suspension was transferred to a 100mm plate. G418 was added to the plate the following day.

# CHAPTER III

## RESULTS

### Triton Cytoskeleton Myosin II and Actin Localization

The purpose of this study was to determine the effects of the CC domain of MHK-A on F-actin and myosin II levels between cytoskeleton and cytosolic fractions within the cell. The purpose of these experiments was to quantify the levels of myosin II and actin in the triton-resistant cytoskeleton and to estimate the levels of filamentous actin and assembled myosin II levels in the cell. For this study, five cell lines were used: Ax2, MHK-A null, MHK-A null + GFP-CC, Ax2 + GFP-CC, and Ax2 + GFP-C800A. To confirm the cell lines, a Western blot was performed using an anti-MHK-A primary antibody and a goat anti-rabbit secondary antibody (Figure 4). All cell lines exhibited the necessary protein bands. Ax2, Ax2 + GFP-C800A, and Ax2 + GFP-CC cell lines exhibited an endogenous MHK-A band at approximately 127 kDa. The Ax2 + GFP-C800A cell line also included a GFP-C800A band at approximately 154 kDa. An unknown band was present between the 100 and 75 kDa markers for the Ax2 + GFP-C800A cell line, indicating a probable breakdown product of C800A. Cell lines expressing the GFP-tagged CC domain of MHK-A (GFP-CC), Ax2 + GFP-CC and MHK-A null + GFP-CC, exhibited a band at approximately 82 kDa. Comparison of the endogenous MHK-A and expressed GFP-CC or GFP-C800A bands indicate that both fusion proteins are expressed at least 6.5 times the level of endogenous MHK-A.

Cells from all cell lines were fractionated using the triton cytoskeletal ghost

isolation protocol to produce cytoskeleton-enriched pellets and cytosolic

supernatants. These samples were then analyzed via SDS-PAGE and coomassie blue

staining to show the amounts of myosin II (~243 kDa) and actin (~43 kDa) present in

the pellet (P) and supernatant (S) fractions (Figure 5). The amount of myosin II and

actin present was quantified using Scion Image software by measuring the bands'

areas and pixel intensities. Results from the experiments were graphed (Figure 6),

which shows the total percent of myosin II present in TX-100 cytoskeleton pellet

fractions from each cell line. In cells of the Ax2 cell line, 44.4% of the total myosin

II was present in the pellet. The MHK-A null cell line had 64.9 % total myosin II

present in the pellet, a measurable increase from the wild-type cells. Over-

expressing the CC domain of MHK-A within MHK-A null cells also resulted in a

measurable increase in pellet myosin II levels as indicated by the 63.2% total myosin

II found in the MHK-A null + GFP-CC cells. However, over-expressing the CC

domain in MHK-A null cells does not appear to have a significant effect when

comparing the MHK-A null and MHK-A null + GFP-CC cells. The Ax2 + GFP-CC

cells, with endogenous MHK-A and the over-expressed CC domain of MHK-A,

exhibited 59.6 % total myosin II in the pellet, a significant increase from the Ax2

wild-type cells. The Ax2 + GFP-C800A cell line exhibited the least amount of

myosin II in the pellet with only 37.9% being present.


**Labeling of Cells for Visualization of F-actin and Myosin II**

Although the Triton Cytoskeleton Ghost Isolation Assay allows for the

quantification of the levels of myosin II and F-actin within the cell, it does not allow

for the visual localization of the two in the context of the whole cell. Confocal microscopy was employed to allow for this visualization and to further understand the effects of the CC domain, if any, on the localization and reorganization of myosin II and F-actin within the cytoskeleton. Because the CC domain has been shown to bind and bundle actin filaments, as well as inhibit myosin II from binding to F-actin *in vitro*, I predicted that over-expressing the CC domain in *Dictyostelium* cells would lead to a significant and visually detectable reorganization of myosin II and F-actin in the context of the whole cell. Specifically, I hypothesized that myosin II would be unable to bind to cortically-localized F-actin as a result of the CC domain inhibitive properties, thus reducing cortically-localized myosin II.

For this study, Ax2 and Ax2 + GFP-CC cells lines were generated and stained for myosin II and F-actin using an anti-myosin antibody and Texas-Red phalloidin, respectively, as described in the protocols outlined in the Methods section. The slides were analyzed and photographed using 40x oil immersion magnification. Photographs of cells stained for myosin II were taken at various sections within the cell (Figure 9). Mid-section micrographs revealed that the levels of cortical myosin II were essentially equal for both cell lines. However, further analysis from photographs taken at the bottom of the cells, where the cells are attached to the coverslip, showed increased amounts of cytoplasmically-localized myosin II aggregates (clumps) in the Ax2 + GFP-CC cells in comparison to the Ax2 cells. Figure 9 shows the photographs from the bottom of both cell lines.

Ax2 and Ax2 + GFP-CC cells phalloidin-stained for filamentous actin were analyzed in the same manner. Confocal images of the cells were taken in the middle

and at the bottom of the cells (Figure 10).  Both the mid-section and bottom images

indicate that there is slightly more cytoplasmic F-actin in the Ax2 + GFP-CC cells

than the Ax2 cells.  Furthermore, the actin appears to be more aggregated in the CC

cells, indicating that the CC domain may be cross-linking and bundling the actin *in*

*vivo*.


**<u>GST Fusion Protein WD-repeat and CC Domain Interaction Assay</u>**

The purpose of this experiment was to determine if an interaction between

the CC and WD repeat domains of MHK-A exists, thereby providing a logical

mechanism for its regulation.  The assay involved "pulling down" a GST fusion

protein with the WD-repeat domain after being incubated with purified CC lacking a

GST tag.  Prior to the "pull-down" assay, both GST-WD and CC proteins were

purified from bacterial cells according to the fusion protein purification protocols

outlined in the Methods section.  The purification of the GST-WD protein was

confirmed (Figure 7a) as indicated by the presence of a band at approximately 49

kDa.  The gel also shows the presence of another band located between

the 50 and 75 kDa markers.  This band likely represents the GroEL protein.  The

GroEL protein is a co-purifying contaminant, but it does not interfere with the

activities of purified GST fusion proteins (Rohman and Harrison Lavoie, 2000).  The

purification of the CC protein lacking GST was confirmed (Figure 7b) as indicated

by the presence of a band at approximately 56 kDa.  All bands present below the CC

protein band are breakdown products recognized by the MHK-A primary antibody.

Glutathione agarose bead-associated and soluble fractions from the GST "pull-down" assay were analyzed via SDS-PAGE/ coomassie blue staining and Western blot. It is important to note that initial interaction studies resulted in ambiguous conclusions due to the high background levels present on the Western blots. To minimize the background, the original protocol was changed to include 0.02% BSA (2 μg/ml) in each of the pull-down mixes. The Western blot analysis results for the GST-WD and CC interaction assays (Figure 8) depict the amount of CC protein in the bead-associated pellets and supernatants for all three pull-down mixes: GST-WD + CC, GST + CC, and CC alone. The incubation of GST with the CC domain only was included to ensure that the GST tag has no effect on any possible interactions. As Figure 8 shows, CC is only present in the supernatant fractions for each reaction. Because the GST-WD protein is "pulled down" in the bead-associated fraction, the absence of CC in the pellet indicates that the CC domain of MHK-A does not interact with the WD repeat domain.

# CHAPTER IV

# DISCUSSION

*Dictyostelium discoideum* is a primitive, eukaryotic organism that relies on myosin II contraction of actin filaments for cytokinesis, migration, and other important cellular processes (Kessin, 2001; De la Roche et al., 2002). Because of this, studies of *Dictyostelium* have significant implications for higher eukaryotes, particularly humans, in understanding myosin and actin activity and regulation in a non-muscle cell context (Sasaki et al., 2002). Although further studies are still necessary to make direct correlations between this social amoeba and higher eukaryotes, research has allowed for a deeper understanding of how important cellular processes occur within *Dictyostelium*. The organism relies on a system of myosin heavy chain kinases to negatively regulate myosin II bipolar filament assembly (De la Roche et al., 2002). Bipolar filament assembly is essential for myosin II to contract actin filaments. The MHC kinases are responsible for phosphorylating the MHC tail, thus driving the disassembly and returning the filaments into their monomeric state. When the filaments are in their inactive monomeric state, contraction will not occur.

Three kinases have been identified, with MHK-A being the most extensively studied. With regard to MHK-A, multiple *in vitro* and *in vivo* studies have been conducted to further characterize MHK-A. Cloning of the *mhka* gene revealed a

multiple domain structure consisting of an N-terminal coiled coil region (CC), a catalytic domain, and a WD repeat domain (Futey et al., 1995; Kolman and Egelhoff, 1997). Studies have confirmed that MHK-A does indeed phosphorylate the MHC tail, both *in vitro* and *in vivo*, and it is activated in the presence of F-actin (Egelhoff et al., 2005). Specific studies on the CC domain have shown that this domain is necessary for actin binding and actin-mediated activation of MHK-A. The CC domain can organize actin into bundles and prevent myosin II from binding to actin *in vitro* (Russ et al., 2006). Despite all that is known about MHK-A, many questions are still unanswered: What role does the CC domain play on the reorganization of myosin II and actin within the cell? How exactly is MHK-A regulated? My thesis research focused on addressing these questions.

To address the first question, I performed two different experiments. The Triton Cytoskeleton Ghost Isolation Assay was used to quantify the levels of myosin II and F-actin in cytoskeleton-enriched pellets and supernatants. This method was important in that it would show whether or not the CC domain inhibits myosin II binding to F-actin *in vivo*, evident in reduced levels of myosin II within the pellet (Steimle et al., 2001). Various cell lines were used to determine the CC domain's effect on localization. I predicted, based on previous *in vitro* studies, that over-expressing the CC domain would lead to a shift in myosin II localization from the cytoskeleton pellet to the supernatant with the F-actin remaining in the pellet (Russ et al., 2006). This would result from the CC domain's ability to block myosin II from binding to F-actin (unpublished data). The graph in Figure 6, shows a very

24

different picture.  The Ax2 cell line was essential in the study to show the wild-type,

normal levels of actin-associated myosin II.  The MHK-A null and MHK-A null +

GFP-CC cell lines were included to show the effects the CC domain would have in a

cell line with no endogenous MHK-A.  Even though both showed a significant

increase in cytoskeletal myosin II in comparison to the Ax2 cells, the CC domain had

no apparent effect in the absence of endogenous MHK-A as indicated by the

relatively equal amounts of myosin II in both MHK-A null and MHK-null + GFP-

CC pellet fractions.  One interpretation of these results is that the CC domain acts in

a dominant negative fashion and inhibits the activity of endogenous MHK-A activity

in Ax2 cells.  In this case, the over-expression of the CC domain acts

antagonistically to the wild-type endogenous MHK-A.  Consistent with this model is

the observation that over-expression of the CC domain does not lead to further

increases in cytoskeleton-associated myosin II filaments.  An alternative hypothesis

is that the over-expression of the CC domain leads to increased cross-linking of actin

filaments, which may "trap" myosin II into the Triton-insoluble cytoskeletal fraction.

However, this model does not account for the absence of an increase in myosin II

associated with the pellet fraction in MHK-A null cells over-expressing the CC

domain.

The Ax2 + GFP-C800A cell line was included to show the effects of the CC

domain within a "dead" kinase.  In this case, MHK-A is present but the change at the

800 residue from a cysteine to an alanine renders MHK-A inactive.  Therefore, The

Ax2-GFP-C800A cell line was essential to determine whether or not the CC domain

is accessible for binding F-actin in the context of the full-length kinase.
Interestingly, the Ax2 + GFP-C800A cell line exhibits essentially the same level of
cytoskeletal myosin II compared to that of the Ax2 cells. Because of this, the CC
domain in the Ax2 + GFP-C800A cells appears to be accessible, allowing for F-actin
binding even within the inactive kinase.

The second experiment I performed to address the CC domain's effect on
myosin II and actin localization and organization was confocal imaging of cells
probed with either anti-myosin II antibodies or Texas-Red phalloidin for actin
staining. This method is not very effective for quantitative purposes, but it allows
for visual analysis in the context of the whole cell. Previous *in vitro* studies have
shown that F-actin is localized to the cortex of Ax2 cells with few detectable
aggregates (Lee et al., 1998). Because myosin II binds to actin, myosin II is also
localized to the cortex. However, in CC over-expressing cells the F-actin becomes
aggregated and localized throughout the cell. Based on these studies, I predicted that
*in vivo* studies would produce the same results. Figure 9 shows the results for
myosin II localization in Ax2 and Ax2 + GFP-CC cell lines. Although the
differences are not striking, Ax2 + GFP-CC cells appear to possess more
cytoplasmically-localized aggregates than Ax2 cells. F-actin also appears to be more
aggregated and cytoplasmically-localized in the CC over-expressing cells (Figure
10). Although the F-actin localization is consistent with *in vitro* studies, it was not
expected that the myosin II would still co-localize with the F-actin in cells over-
expressing the CC domain. This is surprising due to the CC domain's inhibitory

effect on myosin II binding with F-actin. However, this is consistent with the results from the triton cystoskeleton ghost isolation assay, which show an increase in cytoskeleton-associated myosin II in CC over-expressing Ax2 cells. Consequently, the cross-linking and bundling capabilities of the CC domain may in effect be "trapping" the myosin II, leading to the appearance of co-localization.

The second question that my research addressed focuses on the mechanisms regulating MHK-A activity. Studies have shown that MHK-A is activated in the presence of F-actin (Egelhoff et al., 2005). However, the mechanism of this regulation is not yet understood. Because the WD repeat and CC domains of MHK-A function in other protein-protein interactions, I hypothesized that they may interact with each other to provide a mechanism for MHK-A regulation. Many proteins are regulated or activated via conformational changes resulting in a transition from a folded or inactive conformation to an open or active conformation. Because of the location of the WD repeat and CC domains (on opposite ends of MHK-A), it seemed possible that they may interact with each other to induce such changes. To test this hypothesis I utilized the GST pull-down assay method to test for protein-protein interactions. I used this method because it is a relatively simple procedure with significant sensitivity and specificity. Figure 8 shows the results of the immunoblot probed with anti-MHK-A antibody performed using the bead-associated and soluble fractions from three reaction mixes representing the GST-WD repeat and CC domains together, GST and CC domain, and CC alone. The figure clearly shows the presence of CC in the supernatant in all reactions.

The most obvious conclusion from the "pull-down" assay data is that the WD repeat and CC domains simply do not interact to regulate MHK-A and that it is not regulated in this "folding" manner. This is certainly plausible considering that very little is known about MHK-A regulation, leaving a multitude of possibilities open for scrutiny. However, it is important to note that the nature of the assay can not conclusively confirm or refute the presence of an interaction. The possibility of an interaction between the two domains still could exist, but in another context. Furthermore, the "pull-down" assay leaves room for possible false negatives. Although using an immunoblot is fairly sensitive, a weak interaction between the GST-WD and coil proteins may not be detectable, leading to a false negative. A negative result could also be caused by protein denaturation, possibly during purification. Also, protein domains could possibly lose activity when they are expressed as truncations or in bacteria. This particular assay included two truncations of MHK-A. It is possible that the WD repeat and CC domains must be present as part of the full-length MHK-A protein to be functional and interact with each other.

In summary, over-expressing the CC domain appears to play a role in organizational changes of F-actin and myosin II within the cell, as indicated by the result of the Triton Cytoskeleton Ghost Isolation and localization studies. However, further research is needed to understand this role and to provide an explanation for the differences between *in vitro* and *in vivo* studies. Also, MHK-A regulation does not appear to be controlled by an interaction between the CC and WD repeat

domains.  Future MHK-A studies need to focus on the importance of the CC

domain's ability to cross-link and bundle actin filaments and whether or not this is

contributing to the seemingly "trapped" co-localization of myosin II with F-actin in

cells over-expressing the CC domain.  With regards to MHK-A regulation, another

protein-protein interaction assay could be employed to confirm the results from the

"pull-down" assay.  More importantly, other possible mechanisms need to be

considered.  Because myosin filament disassembly is regulated by more than just

MHK-A, conducting these experiments on MHCK-B and MHCK-C as well could

shed more light on the questions addressed in this research study.

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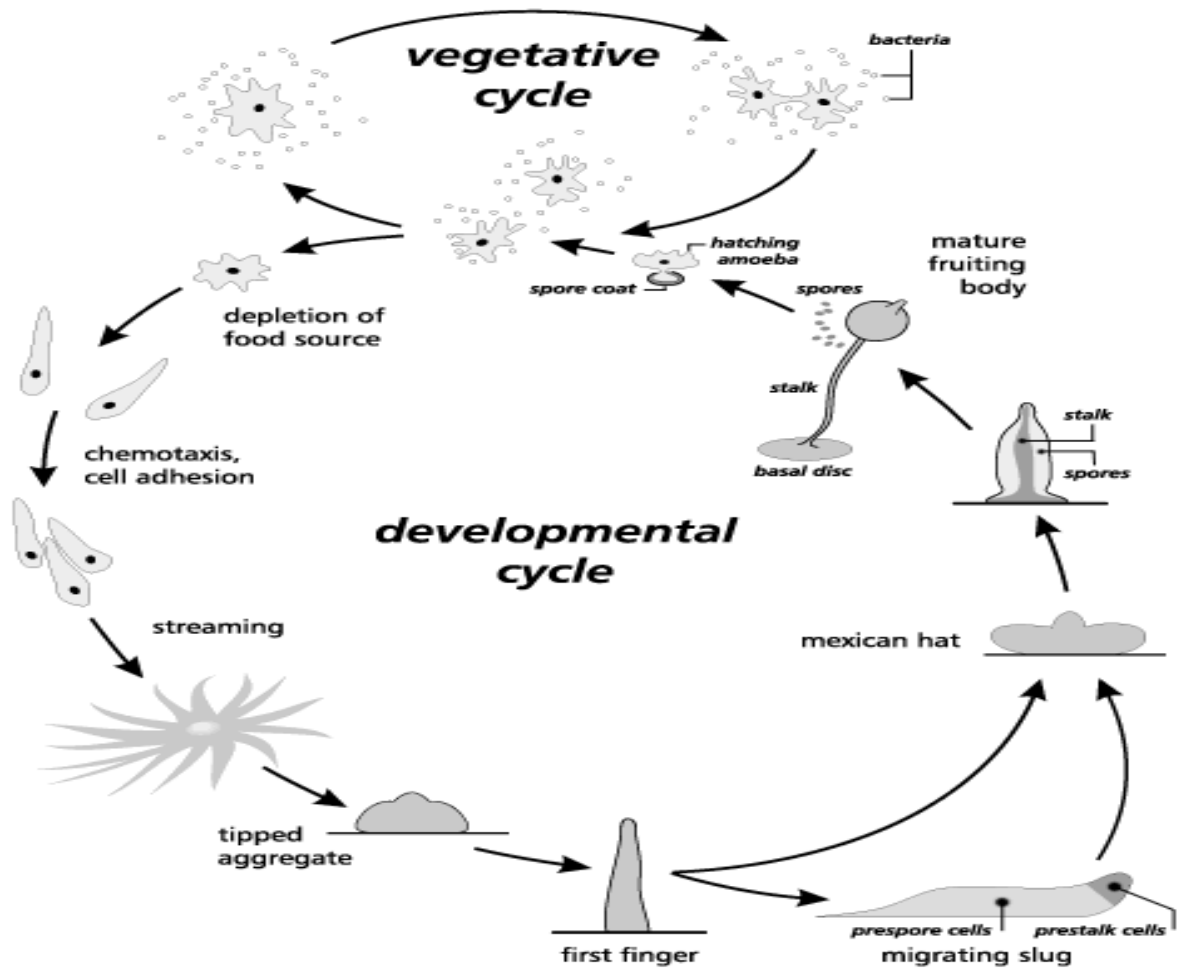
**Figure 1:** The major life cycle stages of *Dictyostelium discoideum* (http://www.dictyostelium.com/devcyc.gif)

No contraction

**Myosin Monomer**

MHC
Phosphatase(s)

**Myosin
Bipolar Filament**

MHC
Kinases

Contraction

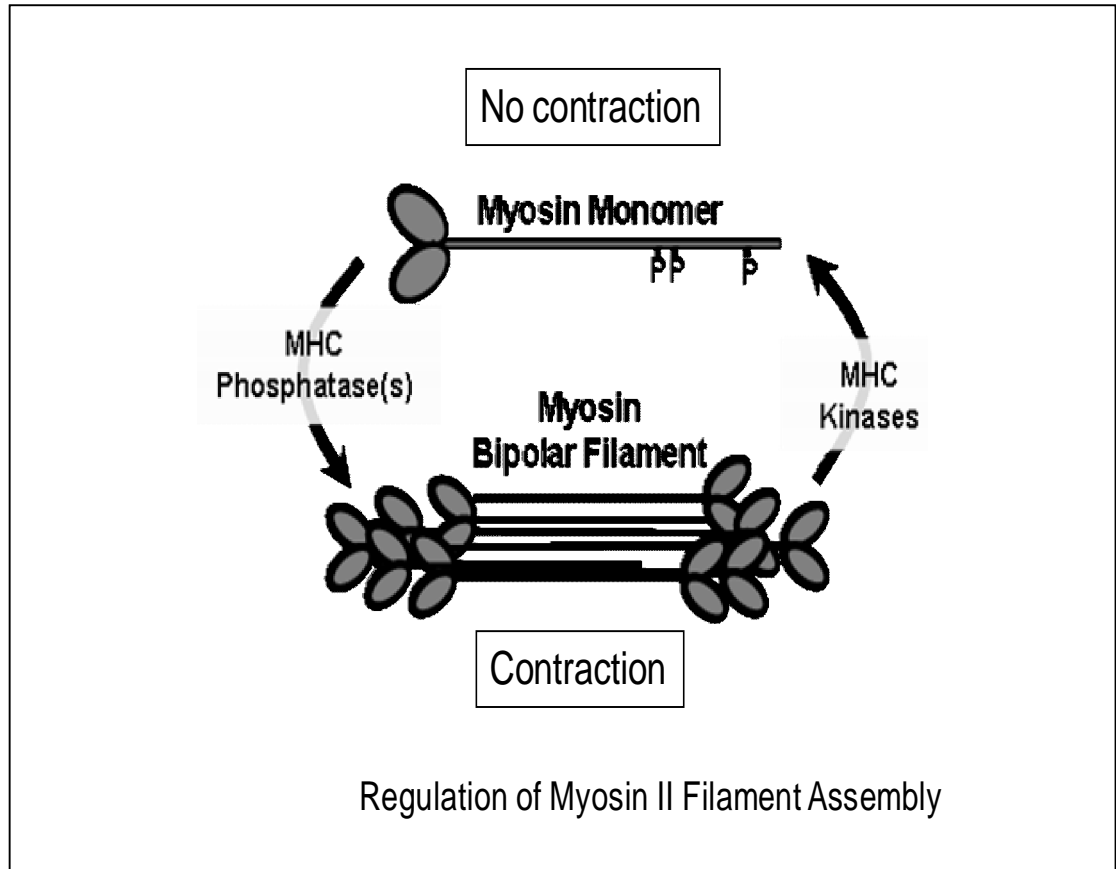Regulation of Myosin II Filament Assembly

**Figure 2**:  **Regulation of myosin II bipolar filament assembly in**
*Dictyostelium*
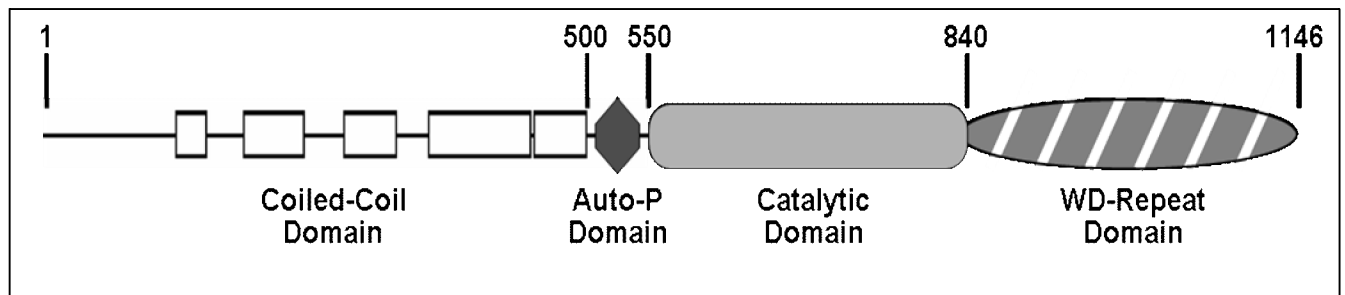
**Figure 3:** **Structural domains of MHK-A. The open blocks represent regionsin the amino-terminal "coiled-coil" domain (1-500 aa) exhibiting a high probability (>0.5) to fold into α-helical coiled-coil structures based upon analysis of the sequence with the COILS algorithm (Lupas, 1996). The dark gray triangle represents the putative autophosphorylation (Auto-P) domain of MHK-A. The central catalytic domain (light gray rectangle) is the prototype for the α-kinase family of novel serine/ threonine kinase. The carboxyl-terminal WD-repeat domain (striped oval) contains a 7-fold WD repeat motif that is characteristic of the ß-subunit of heterotrimeric G-proteins, this domain physically targets MHK-A to phosphorylate myosin II filaments.**
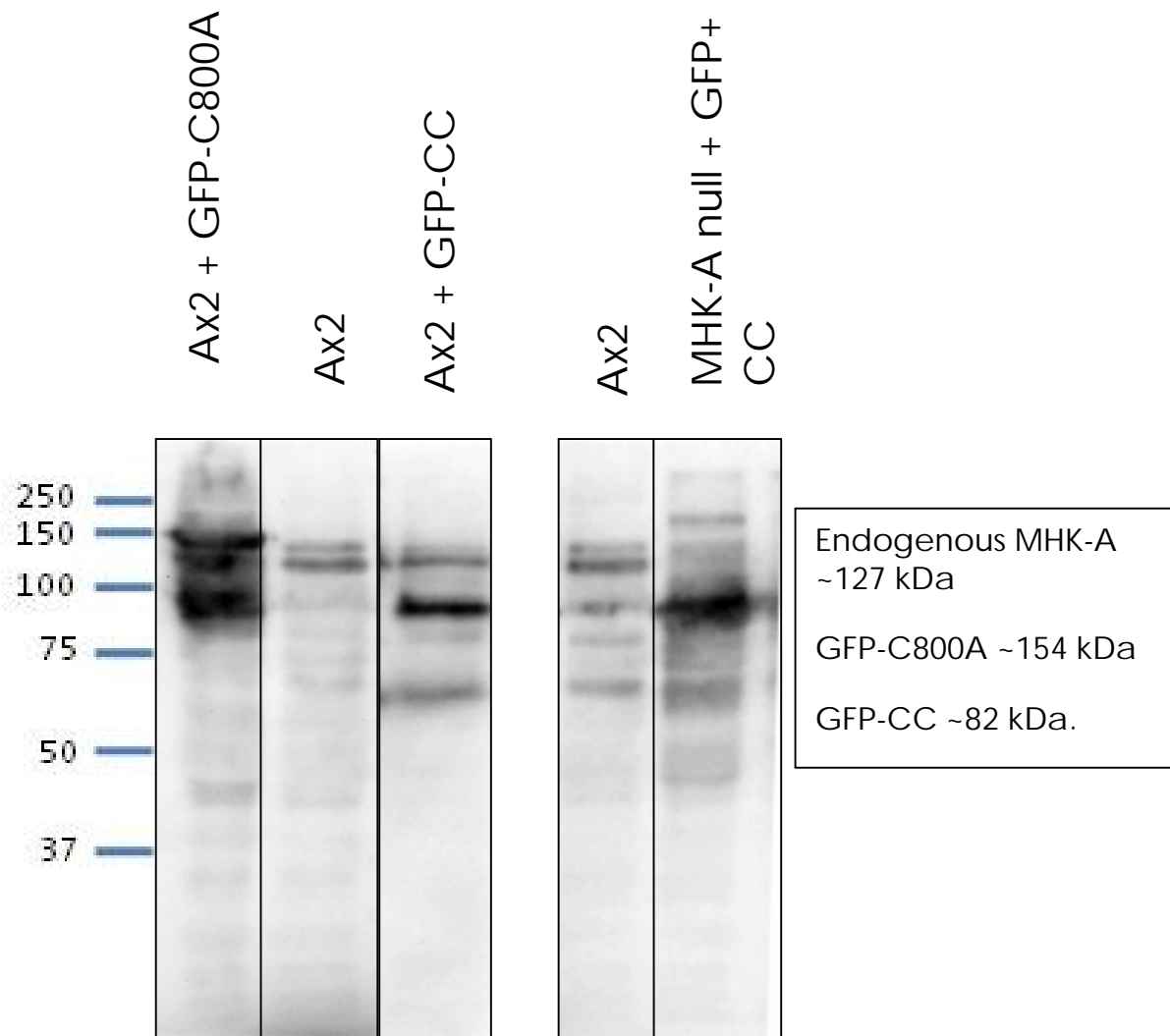
**Figure 4**: **Immunoblots probed with anti-MHK-A antibody to confirm triton cytoskeleton ghost isolation cell lines**
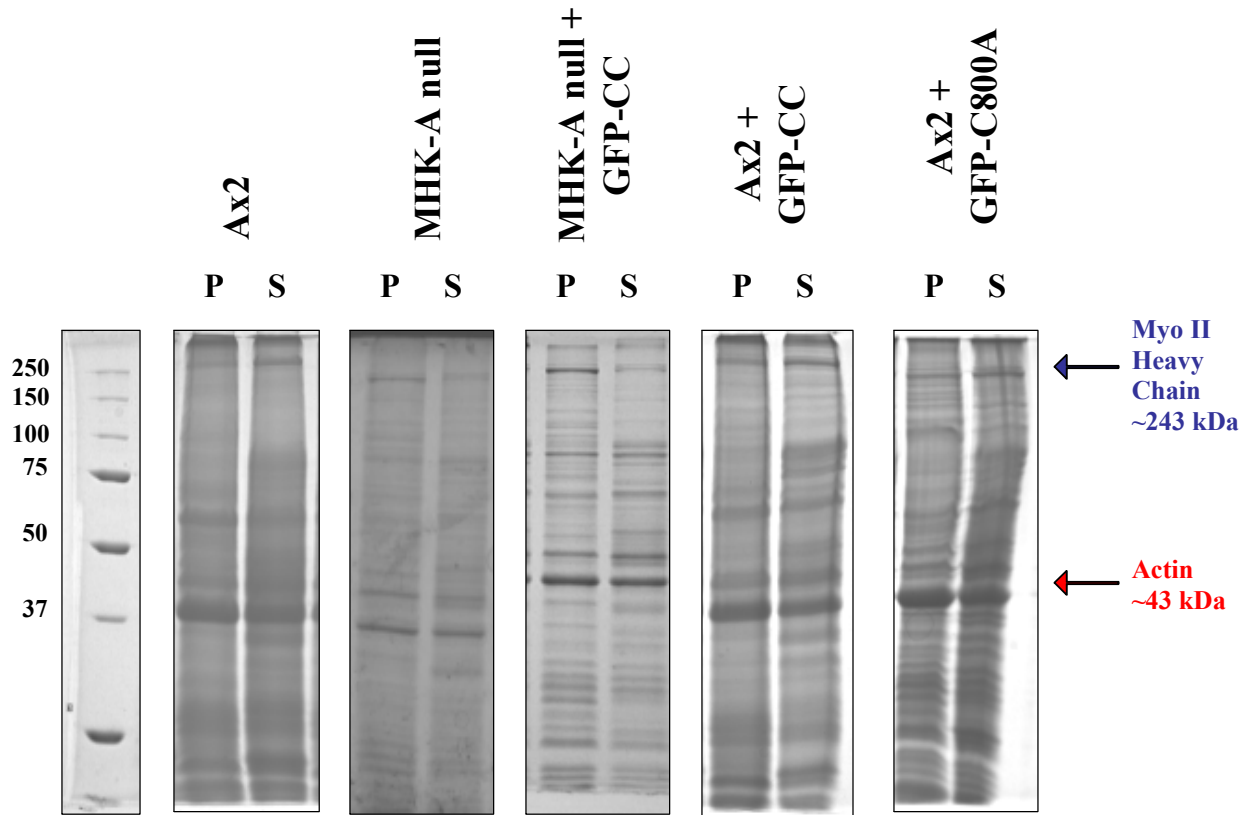
**Figure 5:** Triton cytoskeleton pellet (P) and supernatant (S) SDS-PAGE  fractions to determine levels of myosin II and actin for Ax2, MHK-A null, MHK-A null + GFP-CC, Ax2 + GFP-CC, and Ax2 + GFP-C800A
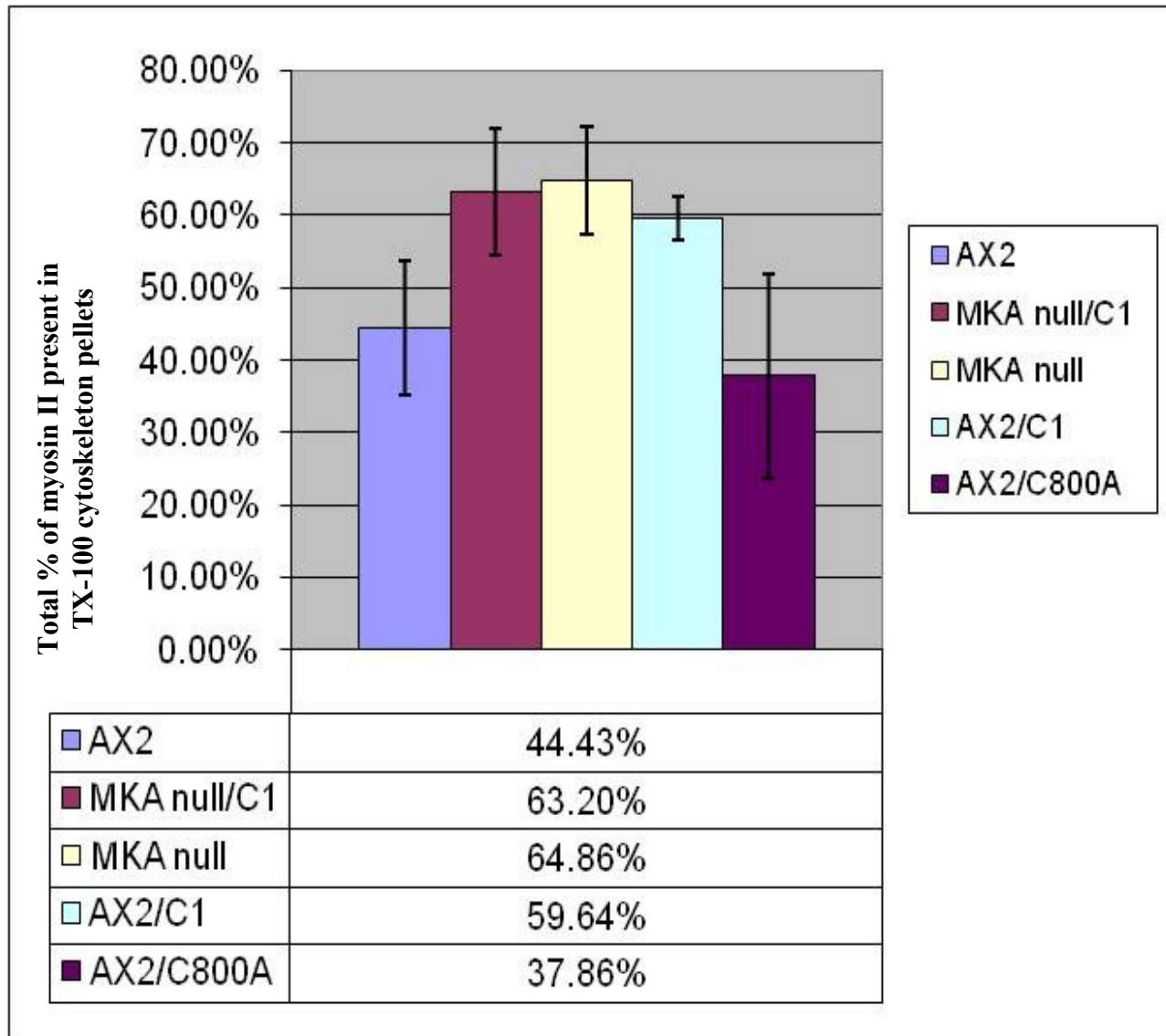
**Figure 6:  Percent total myosin II in TX-100 cytoskeletal pellet
fractions from Ax2, Ax2 + GFP-CC, MHK-A null + GFP-CC,
MHK-A null, and Ax2 + GFP-C800A cell lines**
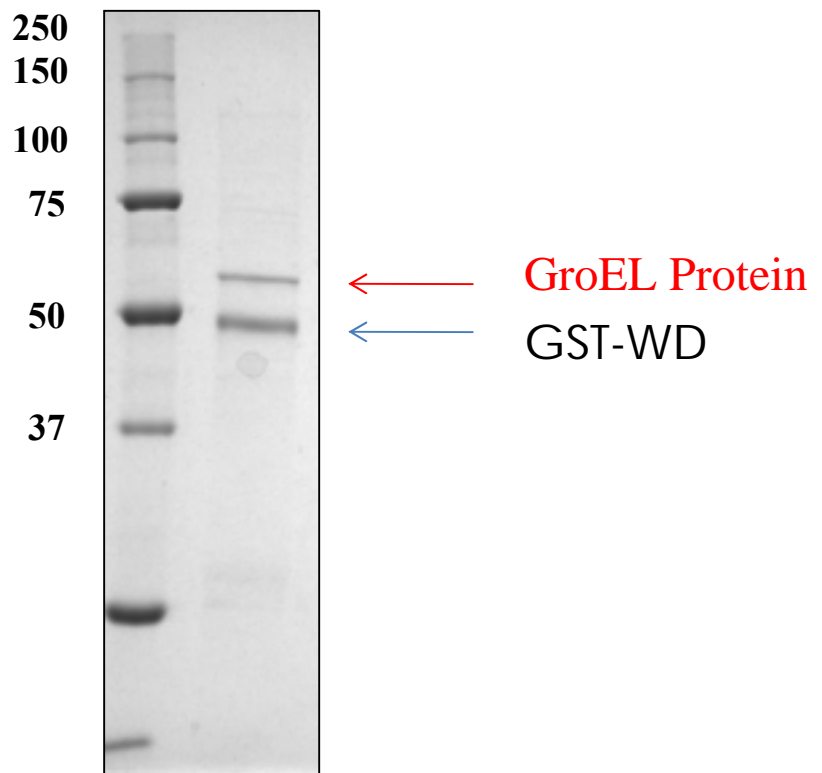
**Figure 7a:** SDS-PAGE confirming purification of GST-WD protein at approximately 49 kDa
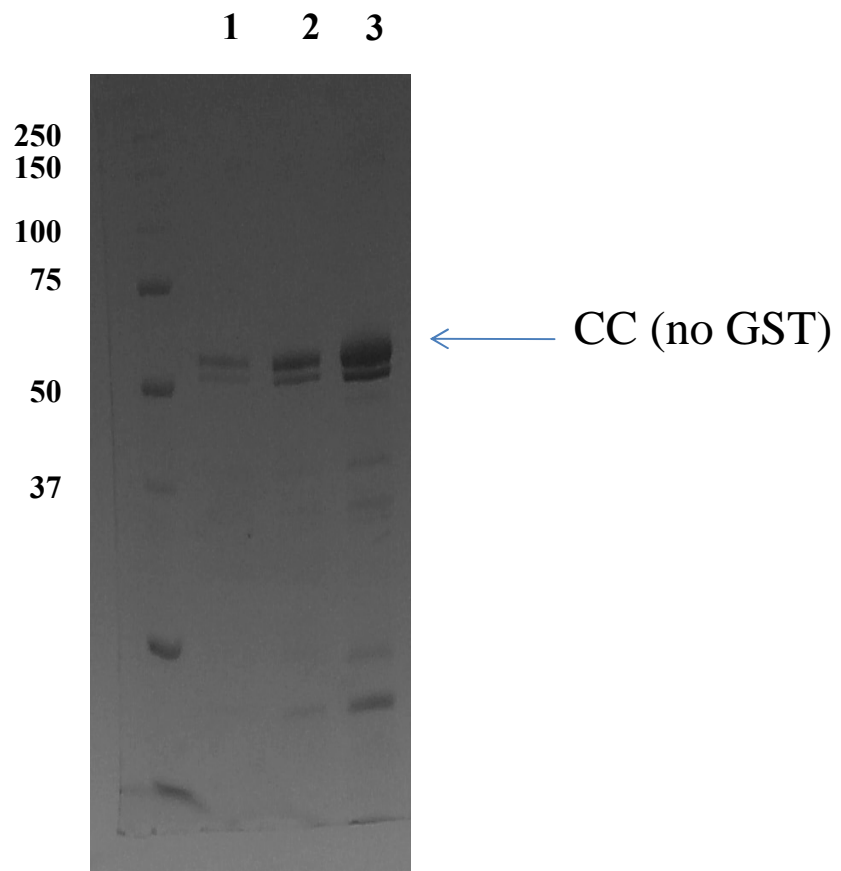
**Figure 7b:** SDS-PAGE confirming CC (lacking GST) protein purification at approximately 56 kDa.

**1.5 μM GST-WD** — P S

**1.5 μM GST** — P S

**No GST Fusion Protein** — P S

250
150
100
75
50
37

← 0.3 μM CC
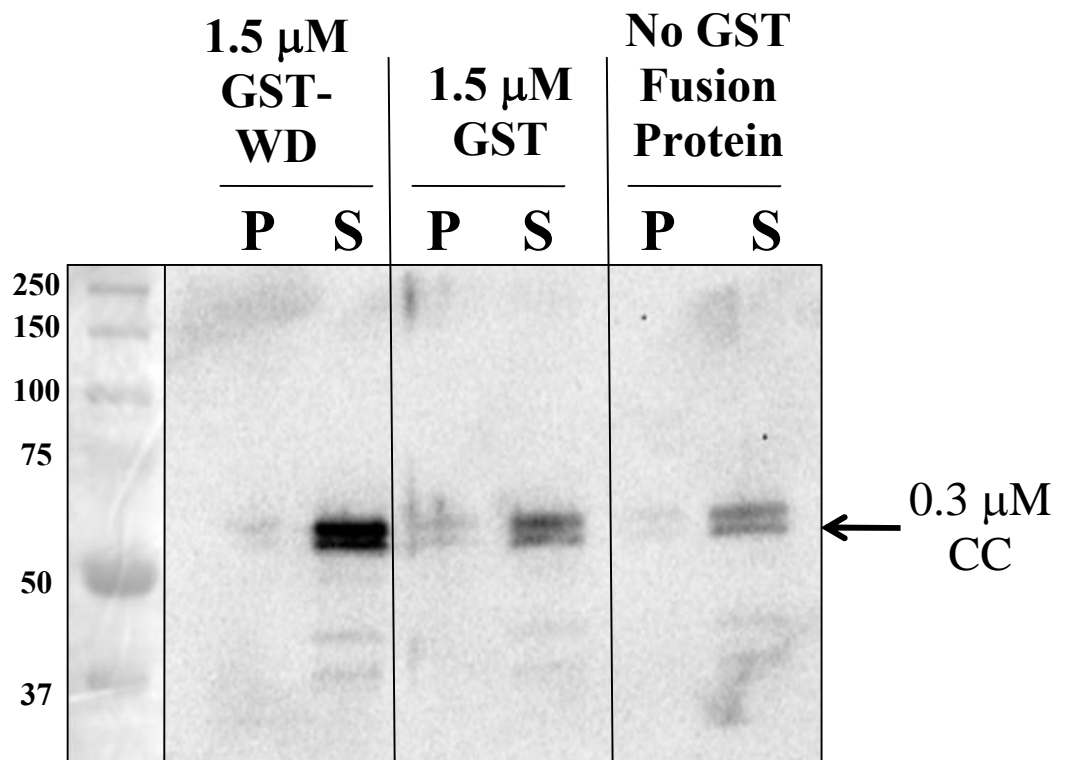
**Figure 8:** **Immunoblot probed with anti-MHK-A primary antibody to determine the presence of an interaction between the WD repeat and CC domains using a "pull-down" assay with GST-WD bead-associated (P) and cytosolic fractions (S)**

# Ax2 Cells Stained for Myosin II

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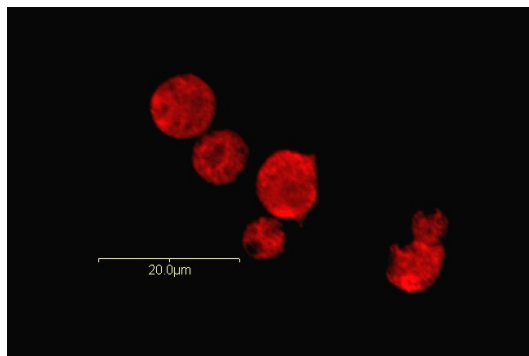
Bottom                           Middle

</div>
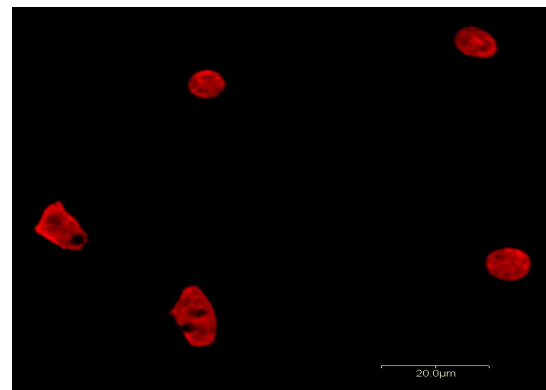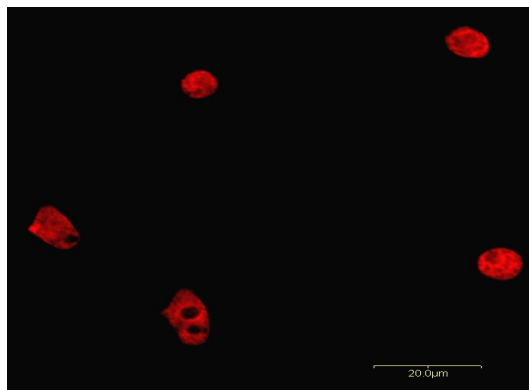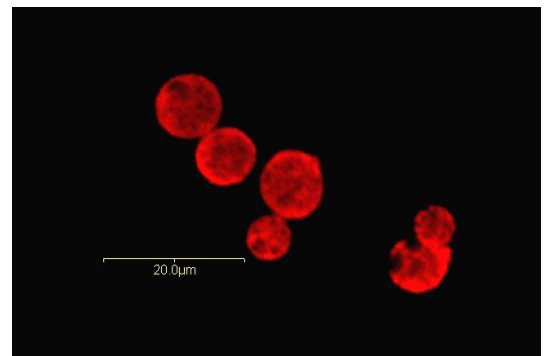


**Figure 9:** **a).Confocal microscopy images of Ax2 cells stained for myosin II with anti-Myosin primary antibody and Alexa-Fluor 546 goat anti-rabbit secondary antibody to visually determine myosin II localization within the cell.**

## Ax2 + GFP-CC Cells Stained for Myosin II
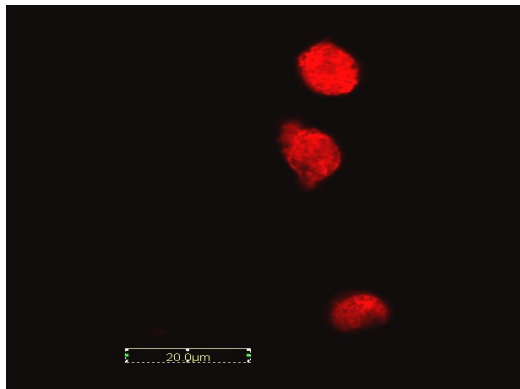
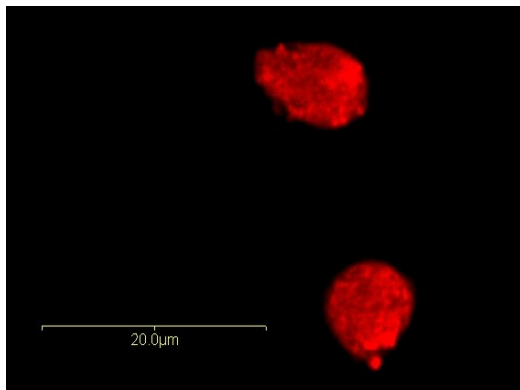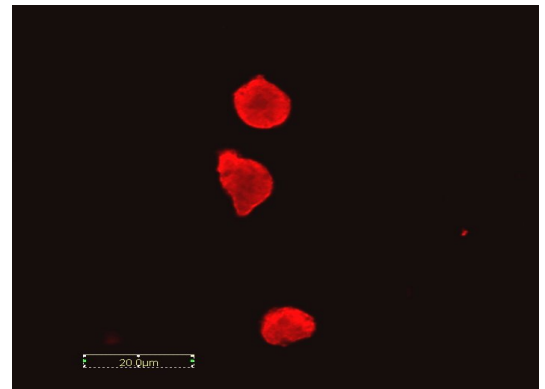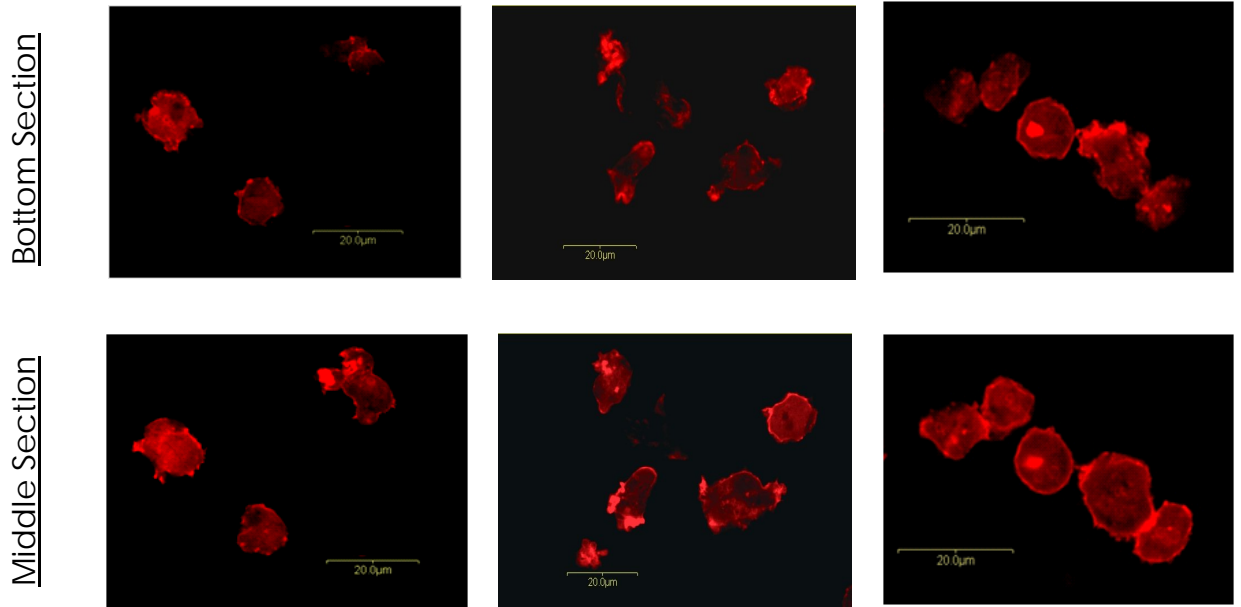Bottom                              Middle
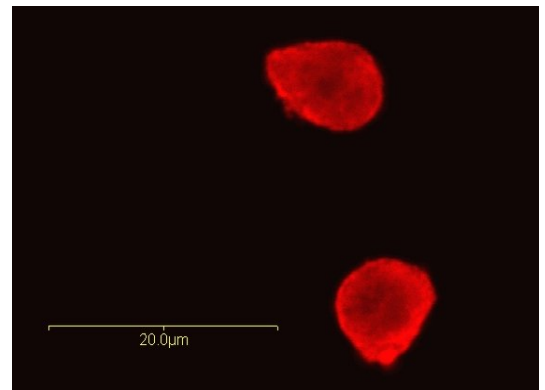


**Figure 10:** a).Confocal microscopy images of Ax2 + GFP-CC cells stained for myosin II with anti-Myosin primary antibody and Alexa-Fluor 546 goat anti-rabbit secondary antibody to visually determine myosin II localization within the cell

**Phalloidin-Stained Ax2 Cells (a)**



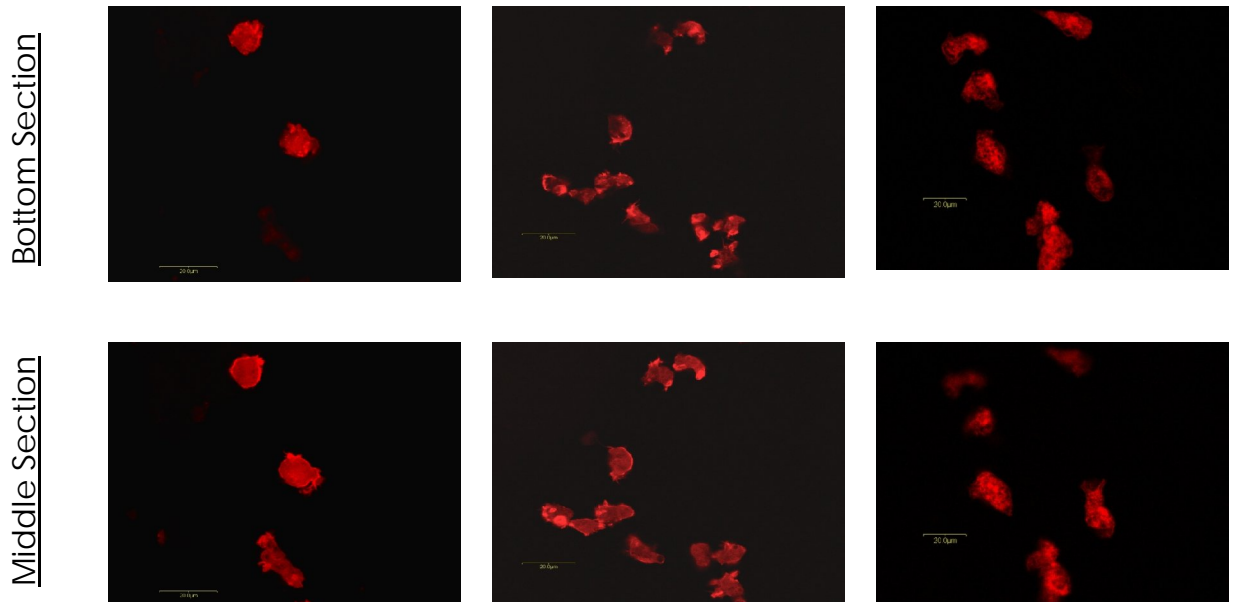**Phalloidin Stained Ax2 + GFP-CC Cells (b)**



**Figure 11: a). Confocal microscopy images of Texas-Red phalloidin-stained Ax2 cells to visually determine actin localization within the cell b). Phalloidin-stained Ax2 + GFP-CC cells**