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Previous studies have shown that myosin heavy chain kinase A and B (MHKA and MHKB) are able to phosphorylate and disassemble *Dictyostelium discoideum* myosin II bipolar filaments by directly targeting myosin II via a conserved region of WD repeats, also referred to as the WD repeat domain. MHKA lacking this domain is unable to phosphorylate myosin II. By comparison, MHKB lacking this same domain is able to phosphorylate myosin II, albeit at a significantly reduced level compared to the full-length protein. The main difference between the MHKA and MHKB proteins is the presence of a region of MHKB in which the amino acid asparagine is repeated 25 times, called the asparagine-rich (pN) region. The function of this region has not been well studied. The research described in this thesis focuses on the asparagine-rich and WD repeat domains of MHKB and their roles in targeting myosin II for phosphorylation. My studies revealed that overexpressing full-length MHKB in *Dictyostelium* cells resulted in slowed growth rates due to cytokinesis defects in which cells become large and multinucleated. MHKB lacking the WD repeat domain (MHKB-ΔWD) also showed this large multinucleated phenotype and slowed growth rate. However, cells overexpressing a truncation of MHKB lacking both the WD and pN regions (MHKB-ΔpN-WD) grew normally and did not become multi-nucleated; indicating that the WD repeat domain is not the only targeting region of MHKB. Triton X-100 cytoskeleton fractionation assays revealed that cells overexpressing full-length MHKB or MHKB-ΔWD have more myosin II in the cytosol than in the cytoskeleton, which is indicative of myosin II being

phosphorylated and disassembled by these two versions of MHKB. By contrast, the myosin II in cells overexpressing the MHKB-ΔpN-WD truncation was located primarily in the cytoskeletal fraction, suggesting that the poly-asparagine region of MHKB may be involved in targeting the kinase to phosphorylate myosin II heavy chain (MHC). Biochemical assays of kinase activity toward myosin II substrate revealed that while full-length MHKB and the MHKB-ΔWD truncation were able to phosphorylate myosin II, the activity of the MHKB-ΔpN-WD truncation toward myosin II substrate was below the limits of detection. This is despite the fact that all three versions of the kinase phosphorylate a peptide substrate to essentially the same level. In summary, MHKB has two major regions involved in targeting myosin II for phosphorylation. The WD-repeat domain is known to directly target myosin II, but is not necessary for the kinase to function. My studies of this system have revealed that the pN region, in combination with the WD-repeat domain, is necessary for MHKB to target myosin II for phosphorylation.

AN ANALYSIS OF THE MECHANISM OF *DICTYOSTELIUM*
MYOSIN HEAVY CHAIN KINASE B IN
SUBSTRATE TARGETTING

By

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APPROVAL PAGE

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CHAPTER I

INTRODUCTION

Dictyostelium discoideum: The Organism

Dictyostelium, a cellular slime mold, is a single-celled eukaryote which lives in the soil and feeds on bacteria. It has two major components to its life cycle. One is the amoeboid stage in which the organism has ample amounts of food and nutrients available, and is often referred to as the vegetative state. Vegetative *Dictyostelium* cells are capable of migrating in a variety of contexts, undergoing mitotic cell division, and exchanging materials with their environment via phagocytosis and exocytosis. When faced with starvation conditions, *Dictyostelium* cells enter into a protective stage of its life cycle that can lead to the formation of three distinct structures: microcysts, macrocysts, or fruiting bodies. The process of microcyst formation is not well known in *D. discoideum*, but it is commonly studied in related species, such as *Polysphondylium pallidum* and involves the production of cellulose. Macrocyst formation is central to the sexual part of the *Dictyostelium* life cycle, and involves the fusion of cells from two mature mating types (1, 2). Environmental conditions favoring macrocyst formation are specific and include conditions of low ionic strength, starvation and submersion.

The formation of the multicellular fruiting body under starvation conditions is a very complex and highly studied process in *Dictyostelium*. *Dictyostelium* multicellular development is initiated when several 'founder' cells in a population secrete cyclic

adenosine monophosphate (cAMP) to other amoeba in the surrounding environment. The surrounding *Dictyostelium* cells respond by migrating toward the source of the cAMP and also by releasing their own cAMP, thus amplifying the signal. In all, approximately 100,000 cells aggregate together by chemotaxis to form a mound (1, 3). The next process in fruiting body development is the slug formation and migration. The slug is a true multicellular state where cells will differentiate into pre-stalk or pre-spore cells. The slug migrates toward light and heat, and once the tip of the slug has found favorable conditions, it will stop moving and continue undergoing development. Continued development involves growth upward to form the final fruiting body structure, and further differentiation of the cells, with approximately 20% of the cells forming the stalk and the rest developing into spores (figure 1). The spores will remain dormant until they are released into a nutrient-rich environment where they will be triggered to germinate and enter into the amoeboid vegetative stage (1).

Dictyostelium in Research: A Model Organism

Dictyostelium has several characteristics which make it a good model organism for studying basic biological processes such as cytokinesis, cell migration, and multicellular development. First, *Dictyostelium* offers many opportunities for manipulation of its genome due to its haploid nature (1). The effects of altering the genome by gene disruption, over-expression, or replacement can be can be monitored via alterations in the various phenotypic characteristics of *Dictyostelium* cells (5). In addition, *Dictyostelium* cells are similar to higher eukaryotic cells in cell structure and

function. For example, they are capable of performing higher functions such as chemotaxis to form cellular aggregates that will ultimately differentiate and form a multicellular structure called the fruiting body (1). By contrast, *Saccharomyces cerevisiae*, a powerful yeast model system for the study of signal transduction and cell cycle regulatory pathways, differ from higher eukaryotic cells, as well as from *Dictyostelium* cells, in that they possess a cell wall which limits its ability to change shape, and thus limits is usefulness as a model system for understanding the basic mechanisms underlying shape changes in the context of the human cell (5). Moreover, the entire *Dictyostelium* genome has been sequenced (6), and genome-wide sequence analyses have revealed overall that there is greater conservation of amino acid sequence among the proteins encoded by *Dictyostelium* and human genes than either of them have with yeast (5). Also, *Dictyostelium* are easy to grow and quick to develop, making them a prime organism for experimentation.

Dictyostelium Cytoskeleton: Composition and Function

Cell movement, cytokinesis, and cell shape depend on changes in the organization of the cytoskeleton. The cytoskeleton is made of actin microfilaments, tubulin microtubles, intermediate filaments, and other supporting proteins, such as anchoring, cross-linking, and capping proteins, as well as motor proteins that drive movement of the cytoskeletal elements or the movement of cargo along filaments and microtubles. In eukaryotic cells actin makes up approximately 8% of the cellular protein and is important for maintenance of cell shape, changes in cell shape, muscle contraction, cytoplasmic

streaming, cell motility and cell division (7, 1). Actin is found in two forms, filamentous (F-actin) and globular (G-actin). G-actin is the monomeric form of actin. F-actin refers to filamentous actin which is formed by the noncovalent assembly of actin monomers and is often bound by supporting proteins, and makes up a large part of the cytoskeleton of the cell. The importance of F-actin in cell locomotion is reflected in the fact that the levels of F-actin increase 50-60% during cell migration. In addition, the assembly and reorganization of actin filaments is critical for the formation of anterior cellular protrusions and for myosin II-mediated contraction at the rear of a migrating cell (1).

Migration of cells involves, in part, the contraction of actin in the cytoskeleton. This contraction occurs by a protein called myosin II or conventional myosin. Myosin II is a motor protein that binds actin and chemically hydrolyses ATP to move forward. Myosin II is a hexameric protein comprised of two heavy chains, two regulatory light chains (RLCs), and two essential light chains (ELCs). The two heavy chains form a coiled-coil tail and the light chains associate with the globular head domain (1, 3). The globular head domain binds to actin and is able to move it by catalyzing ATP hydrolysis. Structurally and functionally, *Dictyostelium* myosin II is very similar to mammalian non-muscle myosin II.

In order for actin to contract, myosin II must assemble into bipolar filaments. The tail region of myosin II associates with the tail region of another myosin II, forming a bipolar filament. The functional myosin II is made of approximately ten to twenty of these assembled myosin II hexamers. In Dictyostelium cells, assembly of the myosin II into filaments is dependent on the phosphorylation state of the myosin heavy chain.

Myosin II contractile activity is regulated by both light chain phosphorylation and heavy chain phosphorylation (1, 3). Light chain phosphorylation activates the motor function of myosin II at the globular head domain. Myosin II Light Chain Kinase (MLCK) phosphorylates the regulatory light chains on serine-13. MLCK-A is the only known MLCK in *Dictyostelium*, though there is evidence of other proteins with MLCK activity since RLC phosphorylation is still detectable in MLCK-A null *Dictyostelium* cells (3, 8). MLCK-A increases the contractile activity of Myosin II four to six fold, but is not required for cellular function of myosin II. MLCK phosphorylation of myosin II does not lead to the formation of bipolar filaments in *Dictyostelium*. Instead, a set of myosin heavy chain kinases (MHKs) have been shown to catalyze the specific phosphorylation of myosin heavy chain and regulate filament assembly (3).

Myosin II has several important functions in non-muscle cells. In general, the major functions of myosin II in non-muscle cells are to drive changes in cell shape and aid in cell movement (9). It is necessary for the 'rounding up' of cells before beginning cytokinesis. Myosin II is involved in the formation of the contractile ring in the cleavage furrow during cytokinesis. It also drives post-mitotic cell spreading and it is capable of rearranging the cytoskeleton. This is important for the release of secretory vesicles. Myosin II is also important in processes such as wound healing. In this process, myosin II is important for contractions that close gaps between cells.

Dictyostelium cell movement and chemotaxis occur through multiple pathways. Install and Andrew suggest that the cell sends out multiple pseudopods and movement occurs in the most favorable direction (10). This is the direction of increasing external

signal cAMP or folate. Location of myosin II filaments during chemotaxis is mainly due to cGMP-binding protein (3). Interestingly, myosin II is not concentrated in the pseudopodia even though movement is occurring. Myosin II is, however, found in the rear of the cell, also referred to as the uropod. Movement of the cell is able to occur via rearrangements of the myosin II in the uropod. Myosin II also keeps the cell polarized so that random turning does not occur during chemotaxis (1).

Myosin II is critical for normal cytokinesis or cell division. Though it was shown that myosin II is not necessary for cell survival since cells lacking myosin II are able to proliferate when cultured on a solid surface. By contrast, myosin II-null cells display a severe cytokinesis defect when grown in suspension culture. Under these conditions myosin II null cells become multi-nucleated and large. When these myosin II null cells were transformed with plasmids containing myosin II, the cytokinesis defect was rescued (1). Therefore, myosin II plays an important role in contracting actin filaments in the cleavage furrow during cell division. Myosin II also plays a role in cell shape. The formation of myosin II bipolar filaments increases the rigidity of the cell via cross-linking actin filaments at the cortex of the cell. In this context, myosin is not required for motor movement, only structure stability (3).

Dictyostelium MHKs are a part of a recently-described kinase family, called alpha-kinases, which differ in protein sequence from the typical threonine/serine phosphorylating kinases. The α-kinase name comes from their preference for phosphorylating their substrates within alpha-helices (11). Moreover, α-kinases differ from conventional serine/threonine kinases in that they exhibit a nearly absolute

preference for phosphorylating threonine residues over serine residues in their substrates. The three known *Dictyostelium* MHKs A, B, and C share not only homologous catalytic domains, but also C-terminal WD repeat domains. It has been shown that the WD-repeat domains of MHKA and MHKB target these enzymes to phosphorylate the myosin II heavy chain by binding directly to myosin II bipolar filaments (12). Functionally, MHKs phosphorylate myosin II, causing it to disassemble into its monomeric, contraction-deficient form (figure 2). Dephosphorylation of myosin II by MHC phosphatases is thought to occur continuously in the cytosol; however, the exact enzyme(s) catalyzing MHC dephosphorylation have yet to be identified. Once myosin II becomes filamentous it will be translocated to the cortex of the cell where it can mediate cell shape change via the contraction of actin filaments. Unlike the presumed MHC phosphatase activity, most of the MHKs become activated upon autophosphorylation. In the case of MHKA, filamentous actin is a potent activator, resulting in a 50-fold increase in MHKA activity (3). In contrast to MHKA, very little is known about the mechanisms by which MHKB and MHKC could be regulated in the cell.

Myosin Heavy Chain Kinase B: Structure and Function

MHKB is one of the three known MHKs (A, B, and C) found in *Dictyostelium* (13). It was first discovered by its homology to MHKA by Clancy et al. MHKB is 733 amino acids long and has a mass of approximately 83 kDa. MHKB has three domains: (1) catalytic, (2) poly-asparagine (pN), and (3) WD repeat domain (figure 3). The catalytic domain is ~250 amino acids with a sequence similarity of 45% to that of

MHKA. The catalytic domain is found in all three MHKs and is responsible for phosphorylating myosin II heavy chain (14). The pN region is unique to MHKB. It is located between the catalytic domain and the WD repeat domain at residues 325-450. Out of the 125 residues of the pN region, 25 are the amino acid asparagine making this region 20% asparagines. These 25 asparagines are in very close proximity to each other and are found between residues 355 and 381. The function of this region is unknown. The WD repeat domain, like the catalytic domain, is found in all three kinases. It is ~260 amino acids long with 43% identity to MHKA (14). The WD repeat domain is a conserved motif of four to sixteen repeats; beginning with GH residues, a variable core of 36-46 amino acids, and ending with the WD residues. The WD repeat domains of MHKA and MHKB target the kinases to myosin II by directly binding to it (12). The localization of MHKB was previously thought to be primarily in the cytosol, however, Yumura et al. has shown that MHKB is enriched in the contractile ring of the cleavage furrow (13). The localization of MHKA and MHKC has been well studied. MHKA is primarily found in the pseudopodia of migrating cells and at the opposite ends of dividing cells. MHKC is localized to the rear of a moving cell and in the cleavage furrow of dividing cells (13, 15).

MHKB, like MHKs A and C, undergoes autophosphorylation. However, unlike the other MHKs, MHKB autophosphorylation does not cause a significant increase in kinase activity towards MH1 peptide substrate. The MH1 peptide resembles the myosin II heavy chain at position 2029 corresponding to the threonine residue, which is one of the mapped target sites for the MHKs in *Dictyostelium*. Experiments using the MH1

substrate have shown that MHKB was able to phosphorylate the substrate with a similar rate as MHKA. *Dictyostelium* MHKB has been shown to phosphorylate myosin II to 1.8 moles Pi/mole MHC and this phosphorylation leads to a reduction of filamentous myosin II from 80% to 24%, in vitro (12). Over expression of MHKB in *Dictyostelium* cells leads to elevated levels of MHC phosphorylation and dramatically decreased levels of functional myosin II bipolar filaments; these alterations in myosin II organization are responsible for the defects in cytokinesis and multicellular development observed in MHKB over-expressing cells. This phenotype is also found in myosin II null cells. Cells in which the MHKB gene has been disrupted by homologous recombination exhibit a significant increase in the level of myosin II bipolar filaments in the cell (11). MHKB null cells, like those lacking MHKA or C, exhibit minor phenotypic defects with cells exhibiting low, but significantly increased levels of myosin II bipolar filament over-assembly. However, myosin II in a recently described cell line in which all three MHKs (A, B, and C) have been disrupted is almost completely in the bipolar filamentous state, and these cells exhibit severe defects in cytokinesis; thus, suggesting that the three kinases work together to regulate myosin II activity the cell (12). However, there is virtually no information on the mechanisms by which the activities of the three MHKs are regulated differentially in the cell in response to the same signal (i.e. cAMP stimulation of cells).

At this time, the substrate targeting mechanism of MHKB is not well understood. The WD repeat domain has been shown to bind directly to myosin II and target kinase A and B to their substrate. A similar function has been proposed for the WD-repeat domain

of MHKC. Comparison of the effects of removing the WD-repeat domains of MHKA and B on the ability of these kinases to phosphorylate myosin II heavy chain revealed that removal of the WD-repeat domain of MHKB resulted in a dramatic decrease in MHC phosphorylation, but did not abolish the phosphorylation of MHC completely. By contrast, the activity of MHKA lacking its WD-repeat domain was below the limits of detection, suggesting an absolute requirement of this domain for the phosphorylation of MHC by MHKA (12). The higher level of phosphorylation of myosin II by the MHKB truncation lacking the WD repeat domain may mean that there is another method by which MHKB is targeted to myosin II (11). My research explored the roles that the WD repeat domain and the poly-asparagine region play in targeting MHKB to phosphorylate myosin II.

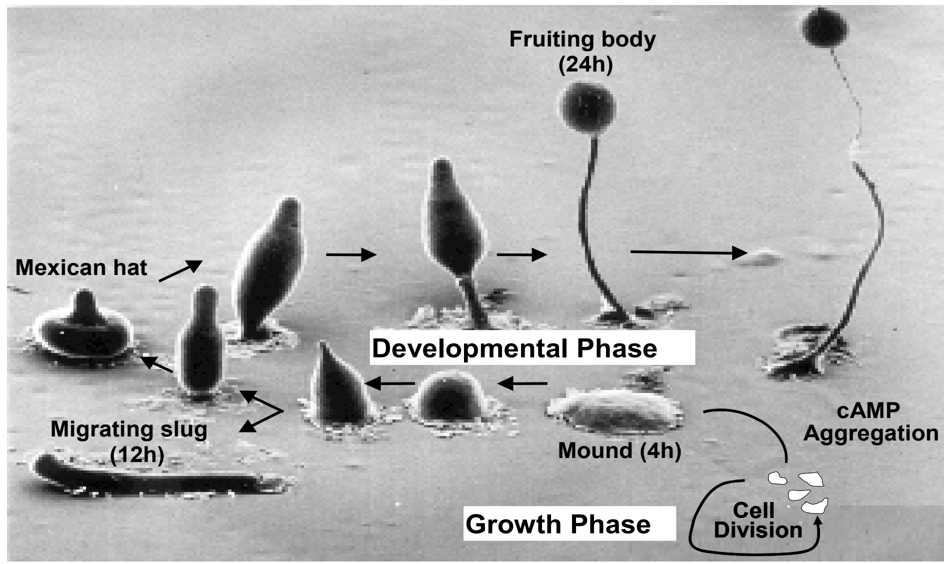


Figure 1: Life cycle of *Dictyostelium discoideum*. Amoeboid cells live freely in the soil and feed on bacteria. Once nutrients are depleted, *D. discoideum* will release cAMP leading to aggregation and mound formation. The cells will then go through several stages in which they differentiate and eventually form the multicellular fruiting body. The fruiting body contains spores, which upon exposure to adequate nutrients, will germinate and release vegetative amoebae into the soil where the cycle will continue [copyright, M.J. Grimson & R.L. Blanton] (4).

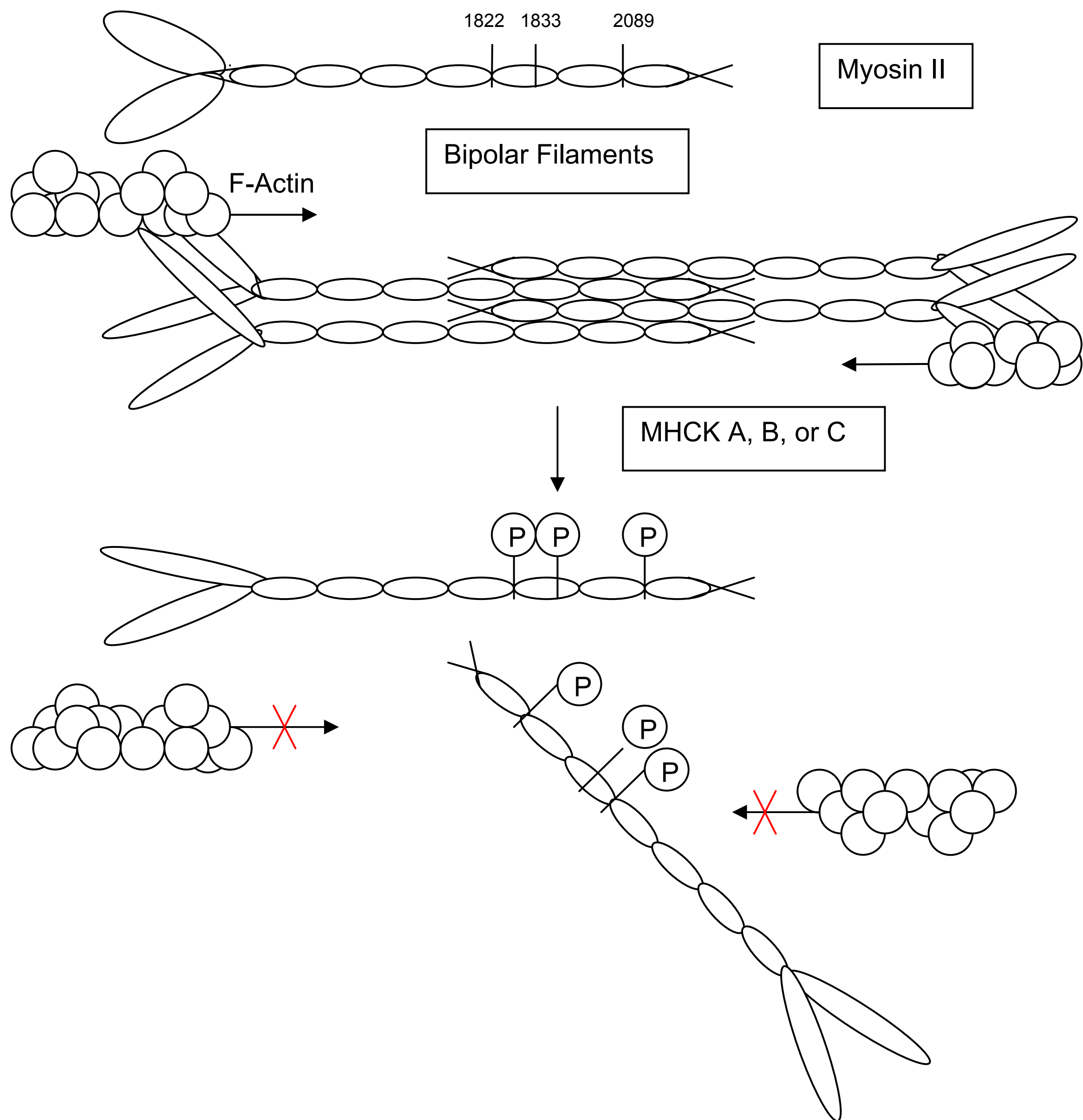


Figure 2: Myosin II Bipolar Filament Formation. Phosphorylation of the Myosin II heavy chain at the three specific threonines at positions 1822, 1833, and 2089 by MHKs leads to bipolar filament disassembly. The monomeric form of myosin II cannot contract actin filaments and is therefore "inactive."

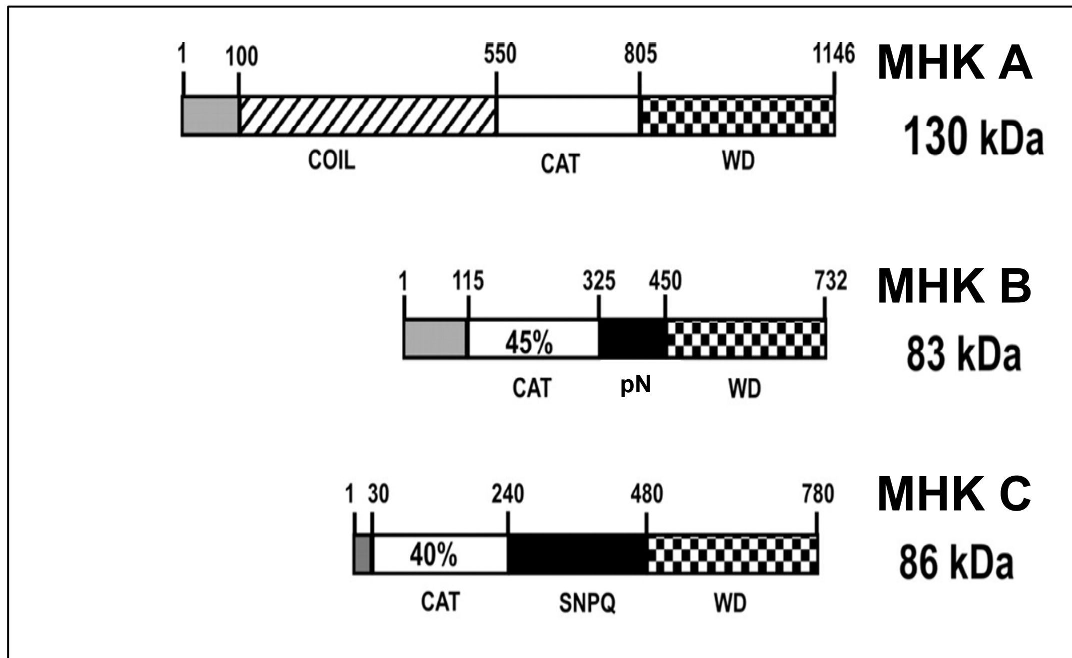


Figure 3: *Dictyostelium* Myosin II Heavy Chain Kinases (MHKs). This figure is a schematic representation of the domain structure of the Myosin II heavy chain kinase family members in *Dictyostelium discoideum*.

CHAPTER II

MATERIALS AND METHODS

Cloning of MHCKB Truncations

The ΔWD and ΔpN-WD truncations of MHKB were generated by PCR amplification from template DNA containing the full-length *mhkb* gene (16). In-frame primers were designed to amplify regions of the MHKB protein encompassing either amino acids 1 to 450 (ΔWD) or 1 to 325 (ΔpN-WD) see Figure 4. The forward primer (5'-GTCGGGATCCATGATATTTAAAGTTTGG-3') contained a BamH1 restriction enzyme site and the reverse (ΔWD: 5'-CTCGAGTAAACAAATACATAAATG and ΔpN-WD: 5'-CTCGAGTAATTCAGATGTATCAGGGG-3') had an Xho1 restriction enzyme site for subsequent subcloning into expression vectors. The PCR conditions were as follows: (1) 95 °C for 1 minute (2) 95 °C for 1 minute (3) 54 °C for 1 minute (4) 72 °C for 1.5 minutes (5) repeat steps 2-4 thirty times (6) 72 °C for 15 minutes. PCR products were gel purified and inserted into TOPO® vector pCR®2.1 per manufacturer's instructions (Invitrogen Corp.) (17, 18). Recombinant pCR®2.1-TOPO® plasmid were introduced into E. coli DH5α cells via chemical transformation (18) and cells containing recombinant plasmid were identified by blue-white screening on Luria –Bertani (LB) agar plates containing 100 μg/ml Ampicillin and 50 μg/ml X-gal. White colonies were selected since they contained the insert and were transferred to tubes containing 3 ml Luria-Bertani broth with 100μg/ml Ampicillin. The liquid cultures were incubated

overnight at 37 °C in a shaking incubator and plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (QIAGEN, Cat. # 27106) (19).

Subcloning of MHKB, MHKB-ΔWD, and MHKB-ΔpN-WD into *Dictyostelium* Expression Vector pTX-Flag

The ΔpN-WD and ΔWD constructs were removed from the recombinant pCR2.1 plasmid by digestion with the restriction enzymes *Bam*HI and *Xho*I (Promega Corp, Cat.# R602A and R616A, respectfully) in a 60μl reaction mix containing the following components: 10 μg recombinant plasmid, 40 Units of BamH1, 40 Units of Xho1, 1X Buffer B (Promega Corp, Cat. # R002A), and 1μg/μl BSA (Promega Corp, Cat.# R396D). The reaction was incubated overnight at 37 °C and the DNA products were size fractionated by agarose gel electrophoresis using 1% agarose. The bands corresponding to the excised inserts (either ΔpN-WD or the ΔWD truncation) were purified from the gel using the QIAGEN Miniprep® gel extraction kit. The gel purified ΔpN-WD or the ΔWD truncations of the *mhkb* gene were inserted into the *Dictyostelium* expression vector pTX-Flag (16) that has been cut with *Bam*HI and *Xho*I using Clonables® ligase system (Novagen Corp., Cat. # 70526-3). A control ligation reaction was run lacking an insert. Ligation reactions were placed in a thermocycler at 16 °C for 30 minutes. Transformed Nova Blue® cells (Novagen Corp., Cat. # 70181-4) with one to five microliters of each ligation reaction and transformants were cultured at 37 °C overnight on LB plates containing 100μg/ml Ampicillin. Transformants containing recombinant plasmid were identified by 1) growing overnight cultures inoculated from several colonies in liquid LB containing 100

μg/ml Ampicillin, 2) isolating plasmid DNA using the QIAprep Spin Miniprep protocol, 3) and digesting the purified plasmid with BamHI and XhoI to remove the insert and the presence (or absence) of the insert was analyzed via agarose gel electrophoresis of the DNA in the restriction digest.

Transformation of *Dictyostelium* Cells with Expression Vectors

For *Dictyostelium* transformations, $5X10^6$ cells were used. Cells in HL5 (2) media with 1X penicillin-streptomycin (P/S) (Sigma-Aldrich, Cat # P0781) were collected and washed twice with 250μl of H50 buffer (20mM HEPES, 50mM KCl, 10mM NaCl, 1mM $MgSO_4$, 5mM $NaHCO_3$, 1mM NaH_2PO_4). On the last wash, cells were resuspended in 100μl H50 buffer. Then, 10μl of plasmid was added to cells and 100μl was placed into 0.1ml electroporation cuvettes (Bio-Rad, Cat. # 1652089). Cells were electroporated at 0.85kV/25mF. Each cuvette was placed on ice for five minutes following electroporation. One milliliter of HL5+P/S was added to the cuvettes and then poured onto 10cm culture plates. Seven milliliters of HL5+P/S was added to the plates containing 1ml of transformed cells. Cell culture plates were incubated at 19 °C overnight. G418, an antibiotic that is used to select for cells containing the gene for neomycin resistance, was added 24 hours after transformation. G418 also helps to maintain stable transfected eukaryotic cell lines. Cultures started at 10μg/ml G418 and were increased to 100μg/ml G418 in order to select for cells with high plasmid numbers. Expression of MHKB was verified by whole cell lysis and immunoblot analysis using anti-Flag epitope antibody (Sigma Corp., Cat. # F3165). For whole cell lysis, 10^7 cells

were centrifuged at 126 x g for 5 min and washed with 1 ml of 10mM Tris pH 7.5. Cells were re-pelleted and the supernatant discarded. Cells were resuspended in 100μl of cell lysis buffer (50mM Tris pH 8, 20mM Sodium Pyrophosphate, 5mM ethylenediaminetetraacetic acid (EDTA), 5mM EGTA, 0.2% Triton X-100, and 2x protease inhibitor cocktail (mini-Complete, Roche Scientific, Ref. # 11 836 170 001). One hundred micro-liters of warmed 5X sample buffer (0.2M Tris pH 6.7, 20% Glycerol, 0.05% Bromophenol blue, and 10mM β-mercaptoethanol, 10% SDS) was added to the lysate, vortexed briefly and incubated at 95 °C for 4 minutes. A 10% SDS-PAGE gel was ran and proteins were transferred to nitrocellulose paper at 90mA overnight or 350mA for 2 hours for immunoblot analysis using mouse anti-flag primary antibody. The blot was developed using BioRad Immunostar® Substrate (Bio-Rad, Cat. # 170-5018) and imaged using the ChemiDoc XRS system (Bio-Rad).

Analysis of Cytokinesis Defects in *Dictyostelium* Cells Over-Expressing MHKB, MHKB-ΔWD, and MHKB-ΔpN-WD

Sterile flasks containing 15mL HL5+P/S were inoculated with cells to a density of $5x10^4$ cells/ml and cells were grown at 20ºC with shaking (170 rpm). Cell growth was monitored by counting cells with a hemacytometer once per day for up to seven days. Cells were collected between five and seven days of growth and then analyzed for multinuclearity (an indicator of a cytokinesis defect) by staining the cells with DAPI and determining the number of nuclei per cell via fluorescence microscopy.

Triton X-100 Cytoskeleton Fractionation of Cells Over-Expressing MHCKB and its Truncations

Cells were collected and counted: 10^7 cells are needed for Triton X-100 cytoskeleton fractionation. Cells were washed in SB (20mM MES pH 6.8, 0.2mM $CaCl_2$, 2mM $MgCl_2$) using centrifugation at 126 x g for 5min. between each wash. Cells were then be washed in Buffer A (0.1M MES pH 6.8, 2.5mM EGTA, 5mM $MgCl_2$, 0.5mM ATP) and $1.5X10^6$ cells were resuspended in 150μl of buffer A and150μl of buffer B (1% Triton X-100, 100mM MES pH 6.8, 2.5mM EGTA, 5mM $MgCl_2$, 0.5mM ATP, 1X PIC). The cells were vortexed briefly, and then centrifuged at 8,000 x g for 1min. at 4°C. Two hundred micro-liters of the supernatant were placed in 700μl of cold acetone to precipitate the myosin II out of the soluble fraction. Precipitated material was pelleted. Twenty-five microliters of 2X sample buffer was added to the pellets. This was incubated in a 95°C heat block for 4 minutes. A 10% SDS-PAGE gel was ran and stained with Imperial™ Protein Stain (PIERCE, prod. # 24615) (11).

Cloning of the ΔpN-WD Truncation for Expression as a GST Fusion Protein in Bacteria

PCR was used to amplify the MHKB- ΔpN-WD region. The PCR product was run on a 1% agarose gel and then extracted using QIAGEN gel extraction kit, as previously described. Next, plasmids containing GST tag (pGEX-4T; GE Life Science) was cut with BamH1 and Xho1, as previously described. The purified MHKB- ΔpN-WD insert and cut plasmid were ligated using T4 ligase (promega). Ligated plasmid and insert was transformed into BL2.1 cells (Invitrogen, Cat. # 70953). Following

transformation, 50µl of cells were plated on LB/Amp plates and incubated at 37°C overnight. The next day, colonies were selected and placed into tubes containing 3ml LB and 3µl of 100mg/ml Ampicillin. These cultures were placed in a 37°C shaker overnight. Verification of plasmid containing the correct insert was done by QIA quick mini spin as previously described. Plasmids were subjected to BamH1/Xho1 restriction enzyme digestion. Digests were run on a 1% agarose gel. Two cell lines containing the correct insert were used for induction of GST-MHCKB- ΔpN-WD (12).

Analysis of Protein Expression in Bacterial Cells

Approximately 10µl of cell culture from the two tubes selected above were placed in two 50ml Falcon tubes containing 15ml LB and 15µl of 100mg/ml Ampicillin. These tubes were placed in 37°C shaker overnight. These cells were tested for expression of GST-MHCKB- ΔpN-WD by adding in 15µl of Isopropyl β-D-1-thiogalactopyranoside (1.0M IPTG) to tube. Prior to adding IPTG, 100µl of cells were collected and kept at 4°C (labeled as un-induced). Three hours post-induction, cells were lysed and analyzed by a western blot using a rabbit anti-GST primary antibody and alkaline-phosphate goat anti-rabbit 2° antibody. BL2.1 cells that are able to successfully be induced were used for large-scale purification of GST-MHCKB- ΔpN-WD (12).

Larger-Scale Induction of GST-MHCKB- ΔpN-WD for Protein Purification

As described above, two drops of inducible BL2.1 cells were added to 15ml LB+AMP as described previously and placed on a shaker overnight. Before induction

with 1M IPTG, 10µl of BL2.1 culture was placed into 250ml of LB and 250µl of 100mg/ml. These cells were placed on a shaker at 37 °C for approximately three hours. Periodically during the last hour, cells were checked spectrophotometrically at OD600 until the OD reading was between 0.6-0.8. Cells were then placed on a shaker at room temperature and 1M IPTG was added to a final concentration of 0.1M. Three hours post induction cells were centrifuged at 3,468 x g. The supernatant was discarded and the cells were placed at -80 °C for 30 minutes or up to four weeks (12).

<u>Purification of GST-MHCKB- Full, ΔpN-WD, and ΔWD from Bacteria Cells</u>

To begin purification of these proteins, cells were thawed on ice. Five milliliters of 1X PBS was added to pelleted cells. The cells were gently resuspended, and 0.025g of lysosyme (MP Biomedicals, cat. #100831) was added. After 10 minutes, 20µl of 0.5M EDTA, 50µl of 20mg/ml in isopropanol of phenylmethylsulphonyl fluoride (PMSF), and 500µl of 10x protease inhibitor cocktail (PIC) was added. Cells were incubated on ice 10 minutes and then placed into a 15ml loose-top culture tube. The cells were broken open by sonication using a dismembranator. Sonication was performed four times for 20 seconds with 30 second incubations on ice between sonications. After the fourth sonication, 1.5ml of the cell slurry was placed into four 1.5ml microcentrifuge tubes. Cells were centrifuged at 25,000 x g for 30 minutes at 4 °C. While the cells were centrifuging, the glutathione-agarose beads were prepared. Five hundred micro-liters of glutathione-agarose beads (50% slurry: Novagen, cat. #70541) was placed in a 15ml Falcon tube. The beads were then washed 5 times by adding 10mL of 1x PBS to the

beads and using a Handee Resin Separator (Pierce PI69710). Once the cell slurry finished centrifuging, the supernatants were added to the 15mL Falcon tube containing the beads and approximately 2mL of 1x PBS. This was placed on a rotator in 4 ℃ for 30 minutes or it can go overnight. After the proteins have bound to the beads, they were eluted using the same Handee Resin Separator used previously to separate the unbound protein from the bound beads. Then beads were washed 8 times with 1x PBS. The slurry were then transferred to a microcentrifuge tube and centrifuged 1 minute in a bench top centrifuge. Next, 300μl elution buffer (10x GST-Elution Buffer, 10x PIC, 0.2M PMSF, 0.5M EDTA) was added to the beads. This mixture was placed on a rotator for 1 hour to overnight at 4 ℃. Next, the slurry was placed in a mini column and centrifuged 1 minute in a table top centrifuge. To quantify the amount of protein purified a 10% SDS-PAGE gel was run with known amounts of BSA in order to make a standard curve. The gel was stained with Imperial™ Protein Stain to visualize the proteins. Quantification of band intensity was performed by using ChemiDocXRS (12).

Analysis of Kinase Activity

Phosphorylation of myosin II and MH1 peptide was performed at room temperature in kinase buffer (10mM TES pH 7.0, 1mM DTT, 2mM $MgCl_2$, and 0.5mM [γ-^{32}P] –ATP with specific activity of 400-800 Ci/mole). Fifty micromoles of MH1 peptide (RKKFGESEKTKTKEF) was used as the substrate for MHKB, MHKB-ΔWD, and MHKB-ΔpN-WD. The amount of γ-^{32}P incorporated into the MH1 molecules were quantified by spotting reactions onto P-81 filters. Filters were placed into 5% phosphoric

acid to stop the reaction. After three seven minute washes, filters were prepped for scintillation counting. This includes placing filters in scintillation tubes with 2ml Scintiverse® (Fisher, Cat. # SX18). Purified myosin II from *Dictyostelium* was used at a concentration of 0.84μM. MHKB phosphorylation of Myosin II was determined by an initial phosphorylation reaction containing 60nM MHKB, 0.84μM Myosin II, 0.5mM ATP, 2mM $MgCl_2$, 10mM TES pH 7.0, 1mM DTT. The reaction proceeded for 5, 10, and 20 minutes at 22 °C. In order to stop the reaction EDTA was added to a final concentration of 20μM. 5X SDS-PAGE sample buffer was added to the reactions. The samples were boiled at 95 °C for 5 minutes and then run on an 8% SDS-PAGE gel. The gel was stained with Imperial Protein Stain for visualization of Myosin II bands. The amount of γ-^{32}P incorporated into the Myosin II was visualized using phosphorimager (Molecular Dynamics). Quantification was performed using ChemiDocXRS and a scintillation counter (12, 20).

Myosin II Sedimentation Assays

Clean centrifuge tubes coated in Sigmacote (Sigma) were used for all reactions. Reaction mixes contained 0.3μM kinase fusion proteins, 1.0 μM *Dictyostelium* myosin II, and 10X kinase buffer (described previously). After allowing one hour incubation at room temperature, 2.5mM TES, pH 7.0, 40mM KCl, 2mM $MgCl_2$, and 5% sucrose were added. Reactions were centrifuged at 13,000 x g in an ultracentrifuge for 15 minutes at 4 °C (12, 20). Supernatants were removed and pellets were re-suspended in buffer described above. 5X SDS-PAGE sample buffer was added and samples were heated at

95 °C for 10 minutes uncovered. Samples were run on 8% SDS PAGE gel and stained using Imperial Protein Stain (12).

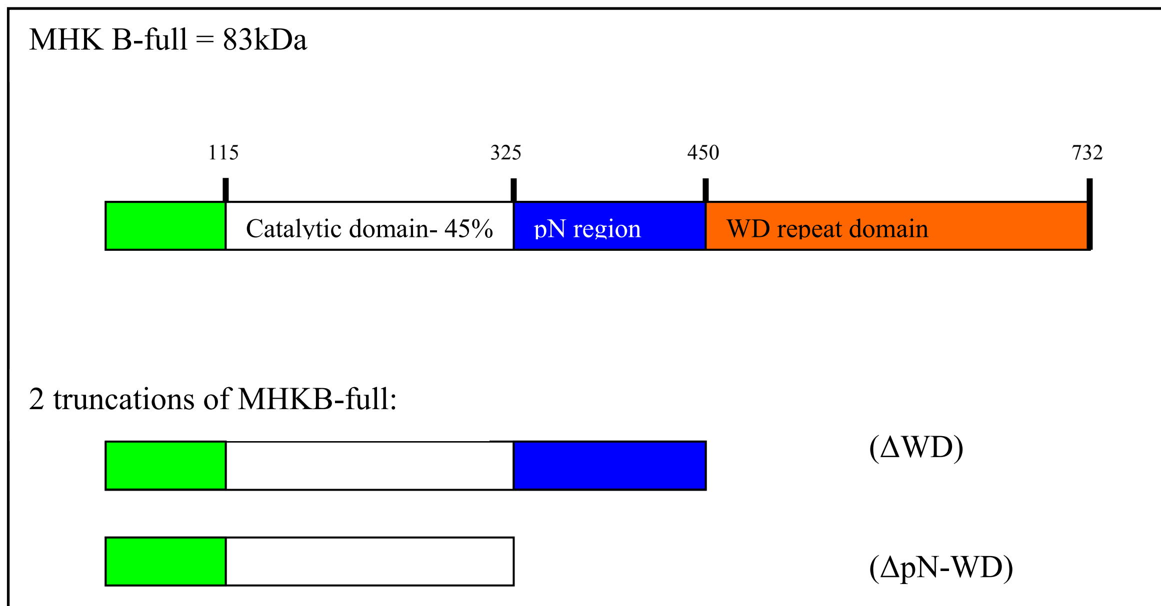


Figure 4: Myosin II Heavy Chain Kinase B (MHKB). The catalytic domain is 45% similar to MHKA's catalytic domain. The first truncated version lacks the WD repeat domain. The second truncation lacks the pN region and the WD repeat domain.

CHAPTER III

RESULTS

<u>MHKB and MHKB-ΔWD Result in Cytokinesis Defects</u>

Dictyostelium cells overexpressing MHKB resulted in a slowed growth rate compared to wildtype Ax2 cells when grown in suspension culture (figure 5). In comparison, cells containing only the catalytic domain are able to divide and grow normally; much like the wildtype (Ax2) cells (figure 5). Interestingly, cells overexpressing MHKB-ΔWD are unable to divide normally and result in a slowed growth rate, resembling cells overexpressing MHKB (Figure 5). In addition, Cells overexpressing MHKB and MHKB-ΔWD both resulted in increased levels of multinucleated cells (figure 6). Cells overexpressing MHKB-ΔpN-WD resulted in 80% normal cells containing 1 nucleus per cell (figure 6). *Dictyostelium* overexpressing MHKB, MHKB-ΔWD, and MHKB-ΔpN-WD resulted in 69%, 63%, and 20%, respectfully levels of multinuclearity, more than one nucleus per cell. Of these, MHKB had ~22% with more than four nuclei per cell, whereas MHKB-ΔWD had ~7%, and MHKB-ΔpN-WD did not have any cells present with more than four nuclei.

<u>MHKB and MHKB-ΔWD Disassemble Myosin II, *in Vivo*</u>

In *Dictyostelium* wildtype cells, less than half (42%) of the myosin II is found in the pellet insoluble fraction when assayed using Triton X-100 Cytoskeleton Fractionation

(figure 7). In cells lacking MHK A, B, and C (A/B/C null cells), well over half (~66%) of the myosin II is in the insoluble pellet fraction (figure 7). This is expected since the A/B/C null cells are not expressing MHKs (A, B, and C), meaning the myosin is not being phosphorylated and thus is found in the filamentous insoluble state. A/B/C null cells overexpressing MHKB resulted in a tremendous decrease (42%) in the pelleted fraction (figure 7) indicating the myosin II is being phosphorylated and disassembled into the soluble fraction. A/B/C null cells overexpressing the ΔWD truncation also showed a decrease (33%) in the pelleted fraction (figure 7). The catalytic domain by itself, overexpressed in A/B/C null cells, closely resembled A/B/C null cells with 64% of the myosin II associated with the pellet fraction (Figure 7). The Coomassie blue stained SDS-PAGE gel clearly shows Ax2 cells have relatively the same amount of myosin II (244 kDa band) in the pellet as in the supernatant (figure 8). A/B/C null cells and A/B/C null cells overexpressing MHKB-ΔpN-WD resulted in most of the myosin II in the filamentous pellet fraction (figure 8). A/B/C null cells overexpressing MHKB and MHKB-ΔWD both resulted in myosin II being present in the soluble fraction (figure 8).

MHKB and MHKB-ΔWD Phoshporylate Myosin II, *in Vitro*

Purified fusion proteins of MHKB showed the highest level of phosphorylation (697.7 CPM) compared to MHKB-ΔWD repeat domain (361.9 CPM) (figure 9). The MHKB-ΔpN-WD was unable to phosphorylate myosin II, though all three kinases showed autophosphorylation occurring. This was not the case with the MH1 substrate. All three kinases phosphorylated MH1 to the same degree (figure 10).

MHKB and MHKB-ΔWD Disassembled Myosin II, *in Vitro*

The full-length MHKB resulted in all of the myosin II being present in the phosphorylated, disassembled state (figure 11). Sedimentation of myosin II with MHKB-ΔWD resulted in approximately half of the myosin II in the disassembled state (figure 11). As seen previously, in the Ghost Triton X-100 Cytoskeleton Fractionation, the catalytic domain by itself resulted in all of the myosin II being present in the filamentous form (figure 11).

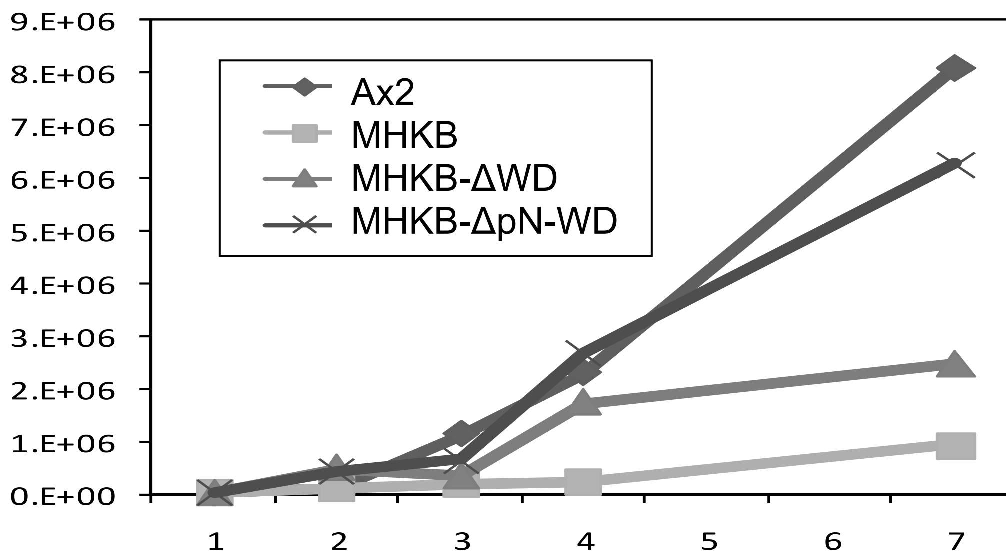
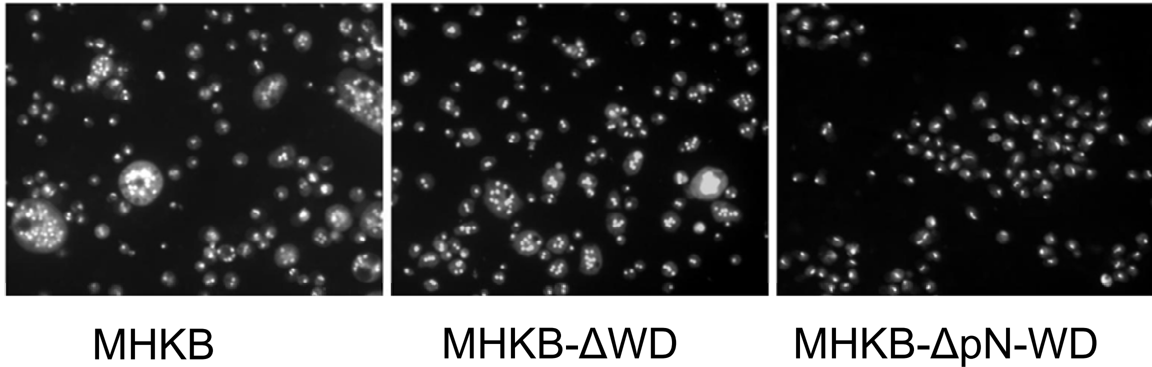


Figure 5: Suspension Growth Curve of *Dictyostelium* Cells Overexpressing Myosin Heavy Chain Kinase and the Two Truncations, ΔWD and ΔpN-WD. Cell counts were taken via hemacytometer 1, 2, 3, 4 and 7 days following addition of 1.0 x 10^5 cells/ml to HL5 media + G418 (0.1mg/ml). MHKB and MHKB-ΔWD resulted in slow growth rates compared to MHKB-ΔpN-WD which resembled wildtype Ax2 growth.

A.



B.

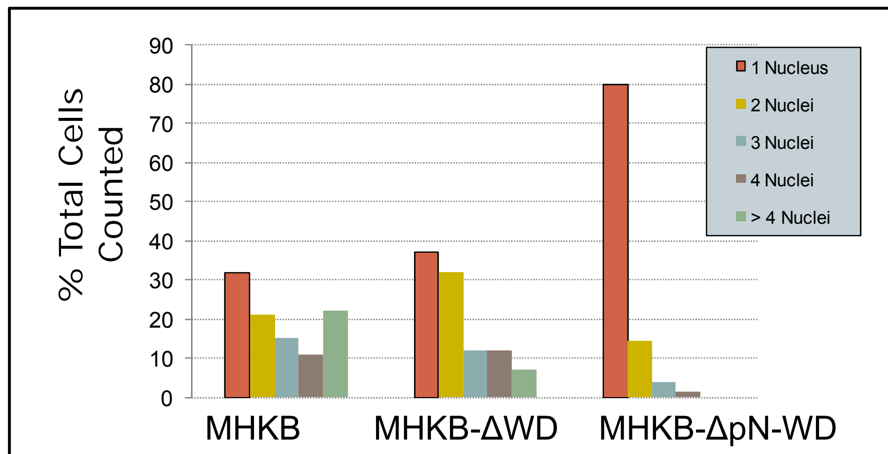


Figure 6: *Dictyostelium* Micrographs and Nuclear Counts. (A) Fluorescent micrographs of *Dictyostelium* cells grown in suspension culture 7days. Fifty thousand cells were used for making slides. DAPI was used to visualize the nuclei. (B) Nuclear counts of the cells depicted in (A). The percent of nuclei present is out of 300 cells total. MHKB and MHKB- ΔWD both showed an increase in multinuclearity; however the MHKB-ΔpN-WD showed normal cell nuclearity, meaning 1-2 nuclei per cell.

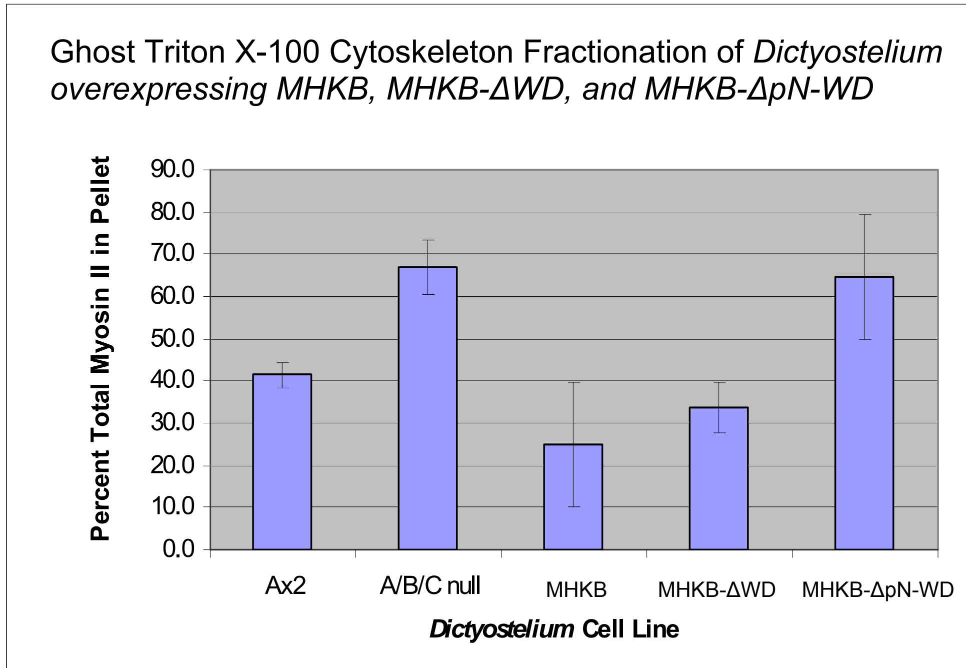


Figure 7: Ghost Triton X-100 Cytoskeleton Fractionation of A/B/C null *Dictyostelium* Cells Overexpressing MHKB, MHKB-ΔWD and MHKB-ΔpN-WD. The percent of Myosin II in the pellet was determined by quantifying both the pellet and the supernatant fragments, then dividing the total (pellet + supernatant) by the pellet fragment. The Full-length MHKB and the MHKB-ΔWD resulted in a significant decrease in filamentous myosin II in comparison to A/B/C null cells, but MHKB- ΔpN-WD did not show significant decrease in filamentous myosin. In fact, the CAT showed more filamentous myosin than wildtype Ax2 cells, closely resembling A/B/C null cells.

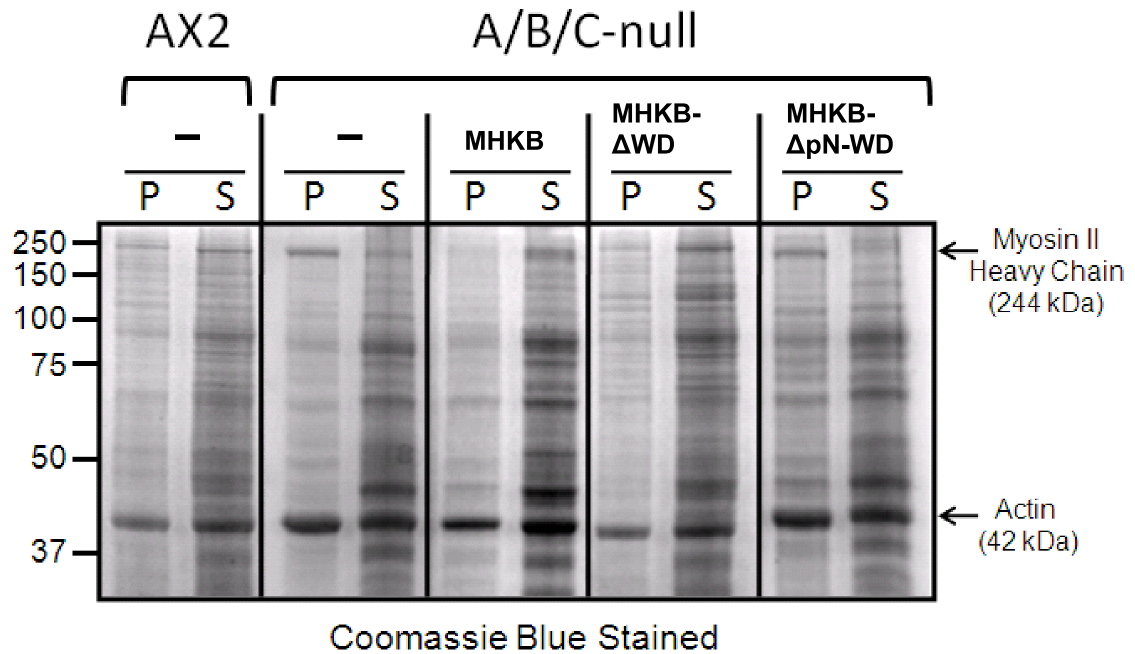


Figure 8: Ghost Triton X-100 Cytoskeleton Fractionation SDS-PAGE gels stained with Coomassie blue. This figure is not of one single assay; however it shows the clearest representative gels of all of the assays performed. Gels are representative of four total assays. *Dictyostelium* cell lines used in the experiment are indicated by brackets and underneath are the expressed kinase. Ghost Triton X-100 Cytoskeleton Fractionation showed the presence of myosin II in either the pellet or the supernatant fragments. MHKB (full-length) and MHKB-ΔWD resulted in most of the myosin II found in the monomeric form, whereas the MHKB-ΔpN-WD had most of the myosin II in the filamentous form.

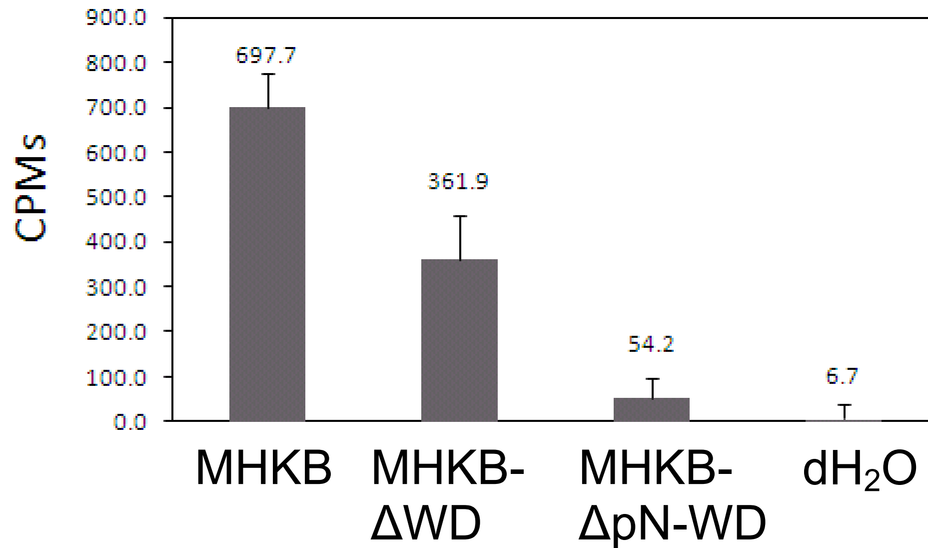


Figure 9: Biochemical Kinase Assay of MHKB and *Dictyostelium* Myosin II. MHKB, MHKB-ΔWD, and MHKB-ΔpN-WD were incubated with myosin II for 15 minutes. Samples were run through SDS-PAGE gel and dried. The myosin II band were cut out of gel and placed in a scintillation counter. The full-length MHKB phosphorylated myosin II at higher level than MHKB-ΔWD, and MHKB-ΔpN-WD. The MHKB-ΔWD construct was able to phosphorylate myosin II, where the catalytic domain by itself showed very little phosphorylation.

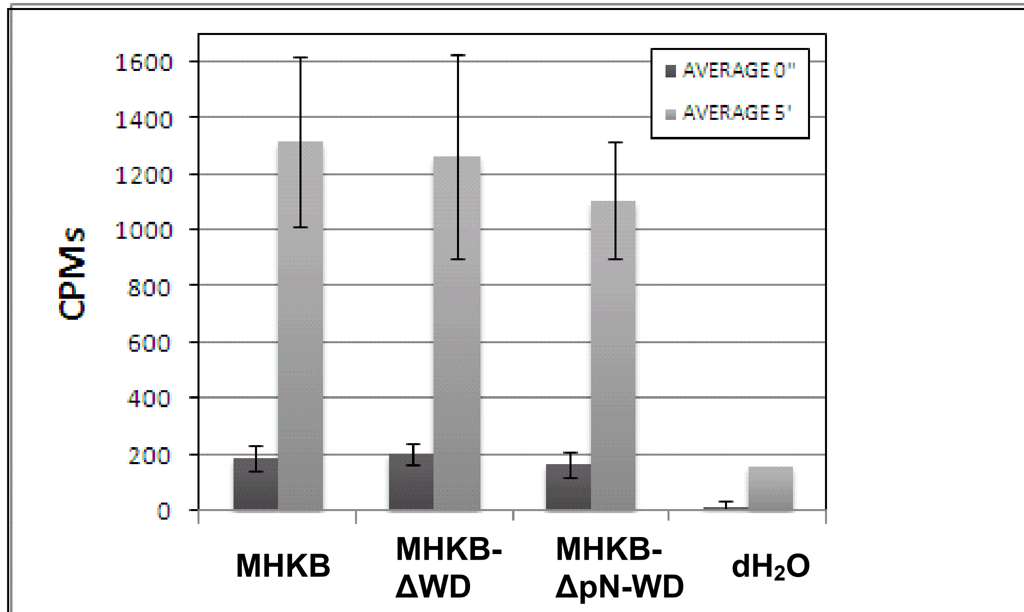


Figure 10: Analysis of Kinase Activity Toward MH-1 Peptide. MHKB, MHKB-ΔWD and MHKB-ΔpN-WD were incubated with MH-1 peptide for 0 and 5 minutes and ^{32}P phosphorylation was detected by a scintillation counter. The standard error bars indicate that all three versions of the kinase are able to phosphorylate MH-1 peptide with relatively the same activity.

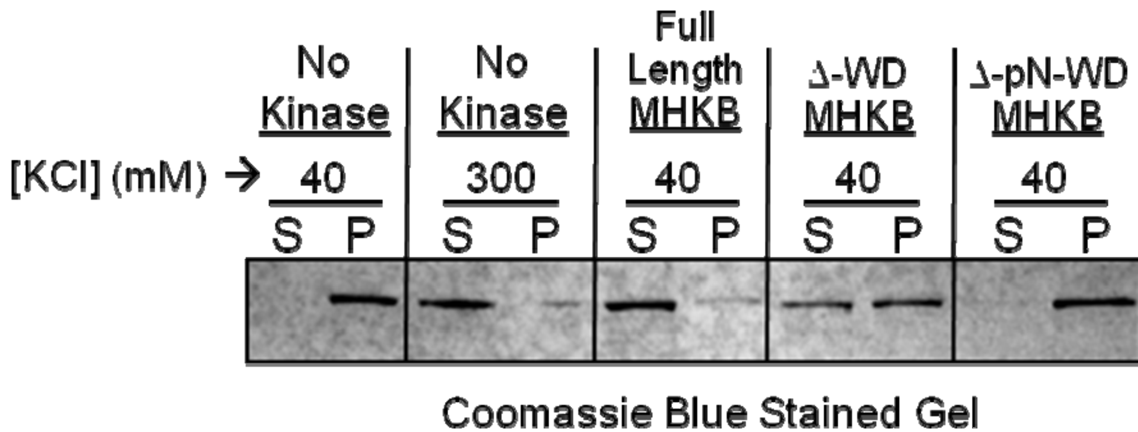


Figure 11: <u>Analysis of Kinase Activity on Myosin II Filament Assembly, *in Vitro*</u>. Purified fusion proteins MHKB, MHKB-ΔWD, and MHKB-ΔpN-WD tagged with GST were incubated one hour with purified *Dictyostelium* myosin II. Samples were subjected to high speed centrifugation and filamentous myosin II resulted in the pellet and monomeric myosin in the supernatant. Samples were ran in 40mM KCl causing myosin II to favor to filament formation. As a control, 300mM KCl was used to show filaments were able to break down into monomers. The positive control lacking the kinase showed that the disassembly of myosin II was indeed due to the kinase.

CHAPTER IV

DISCUSSION

Myosin Heavy Chain Kinase B is one of three known alpha kinases that are involved in the regulation of myosin II assembly. MHKB phosphorylates the heavy chain causing disassembly of myosin II bipolar filaments into its non-functioning monomeric form. The targeting mechanisms of MHKB have not been fully explored. Previous studies have indicated the WD repeat domain is directly involved in targeting MHKA and MHKB to myosin II. These studies also indicated that MHKB lacking this domain had higher levels of phosphorylation of myosin II than MHKA lacking this same region (12). There are two major differences between MHKA and MHKB. MHKA has a coil-coiled region and MHKB has a region rich in asparagines (12, 13). The studies presented in this paper show evidence supporting that not only the WD repeat domain is important for targeting myosin II, but the pN region is involved as well.

The truncation of MHKB lacking the WD repeat domain in conjunction with the truncation of MHKB lacking both the WD repeat domain and pN region has given good insight into the importance of these two regions individually in targeting myosin II. MHKB overexpressed in *Dictyostelium* Ax2 cells has previously been shown to have slowed growth rates and caused a multinucleated phenotype as a result of targeting and disassembly of myosin II (11). Ax2 cells overexpressing MHKB and MHKB-ΔWD displayed slowed growth and an increase in multinuclearity, suggesting myosin II is

targeted for disassembly into its monomeric form by the pN region. It was previously thought that the WD repeat domain was the most important region for targeting myosin II. While it may be for MHKA, it appears as though it is not necessary in the case of MHKB (12).

The results presented in this paper determined that the MHKB is functioning by actually disassembling myosin II. The Ghost Triton X-100 Cytoskeleton Fractionation assays determined that the full-length MHKB and the MHKB lacking the WD repeat domain were both able to disassemble the myosin II bipolar filaments into inactive monomeric myosin II. It is important to note that the full-length MHKB resulted in a slightly higher level of disassembly than the truncated version, suggesting that for full function of the kinase both domains must work together to target myosin II.

In previous studies, overexpression of the full-length MHKB in Ax2 *Dictyostelium* cells indicated that the level of filament assembly did not correlate with the expression level of the kinase, suggesting that there are alternate mechanisms for controlling MHKB activity (11). The low levels of filament disassembly in comparison to Ax2 cells may have something to do with other MHC kinases being present. Previous studies indicate that MHKA, B, and C additively lead to myosin II filament disassembly (13). It is not known exactly how these kinases interact with one another to regulate myosin II filament disassembly. The overexpression of MHKB in A/B/C null cells allowed us to clearly see the level of filament disassembly when compared to A/B/C null cells. In comparison to Ax2 cells, cells overexpressing MHKB did not have a significant level of filament disassembly; however, filament disassembly by MHKB and MHKB-

ΔWD was significant when compared to A/B/C null cells. The catalytic domain by itself did not show significant levels of filament disassembly when compared to A/B/C null cells. Since there are only three major regions of MHKB, it is apparent that the pN region and the WD repeat domain are both important for targeting myosin II for phosphorylation. More studies with other constructs may give insight into how many of these asparagines are necessary to target the MHC.

Autophosphorylation occurs in both MHKA and MHKB. Previous studies show that autophosphorylation does not significantly affect the biochemical activity of MHKB in comparison to MHKA where activity of the kinase is increased 50-fold after autophosphorylation, this suggests alternate mechanisms exist for regulation of its activity (11). MHKA is also activated by F-actin formation, but there have not been any studies to date to determine MHKB activation components (13). Further research should be done to determine how MHKB activity is regulated.

The biochemical assays determined that the pN region and the WD repeat domain both played roles in targeting myosin II. The full-length kinase showed a higher level of phosphorylation of the MH1 substrate than the pN region alone. This suggests that for full activity of the kinase, the pN region and the WD repeat domain work together to target myosin II. The ability of the catalytic domain to phosphorylate the MH1 peptide indicated that the larger myosin II molecule has to be targeted for phosphorylation to occur.

Yumura et al. previously found that MHKB targeted a region on the myosin II tail that was larger than the phosphorylation sites alone (13). Though the experiments

presented in this thesis indicate a novel targeting region of MHKB for targeting of the myosin II heavy chain, it does not give evidence of how this region may be involved in localization of MHKB to the cleavage furrow. More studies need to be performed in order to determine how MHKB is localized and what upstream signaling pathways may be involved.

In vivo and *in vitro* assays indicated that the pN region is sufficient for phosphorylation of myosin II. The WD repeat domain of MHKB is important for directly targeting myosin II, but it is apparent that the pN region is ultimately involved in the kinase's targeting mechanism. However, it is evident that these two regions together are important for optimal targeting of MHKB to the myosin II heavy chain. Future research should investigate the pN region and how its conformation may be involved in MHKB targeting of myosin II and how it may control the cellular localization of MHKB.

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APPENDIX. ABBREVIATIONS

Abbreviations used in Methods and Materials:

BSA = Bovine Serum Albumin

HEPES = N-(2-Hydroxyethyl)piperzine-N'-(2-ethanesulfonic acid)

KCl = Potassium Chloride

NaCl = Sodium Chloride

MgSO4 = Magnesium Sulfate

NaHCO3 = Sodium Bicarbonate

NaH2PO4 = Sodium Phospate

EGTA = Ethylenediamine Tetraacetic Acid

PBS = Phosphate Buffered Saline

TES = N-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid

MgCl = Magnesium Chloride

DTT =1,4 Dithiothreitol

ATP = Adenosine Triphosphate