Regulatory Mechanisms of Myosin I in Dictyostelium discoideum

by

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Abstract

The class I myosins are an ubiquitous family of non-filamentous, single-headed actin-binding motor proteins. The objective of this study was to identify the light chain composition of the short-tailed Dictyostelium class I myosins, MyoIA and MyoIE. Flag-tagged MyoIA head-neck and MyoIE head-neck constructs were generated and expressed in Dicyostelium discoideum. The MyoIA and MyoIE head-neck constructs both co-purified with a 17-kDa protein that reacted with an anti-calmodulin antibody and exhibited a mobility shift on SDS gels in the presence of calcium. Mass spectrometry analysis confirmed that the light chain bound to MyoIA and MyoIE was calmodulin. The finding that the short-tailed class I Dictyostelium myosins use the generic calcium-binding protein calmodulin as a light chain contrasts with previous work showing that the long-tailed Dictyostelium class I myosins MyoIB, MyoIC, and MyoID each bind a unique, specialized light chain called MlcB, MlcC, and MlcD, respectively. Despite having a calmodulin light chain, calcium did not affect the actin-activated Mg-ATPase activities of MyoIA or MyoIE.

The p21-activated kinases (PAKs) are serine-threonine protein kinases that are activated by the small GTPases Cdc42 and Rac. PAKs phosphorylate a site in the motor domain of Dictyostelium class I myosins that is required for myosin activity. Studies were carried out to determine whether Dictyostelium RacB, which is known to bind to and activate Dictyostelium PAKs, promotes the phosphorylation of MyoID in vivo. A vector that expresses a constitutively active RacB under the control of a doxycycline-inducible promoter was created and transformed into Dictyostelium cells. Immunostaining demonstrated that the constitutively active RacB increased actin filament formation in
AX3 cells by ~3-fold but by only ~1.5-fold in PakB-null cells. A rabbit polyclonal antibody against the MyoID tail was made. An anti-phospho antibody raised against a phosphorylated peptide corresponding to the MyoID TEDS site was tested and found to specifically recognize purified phosphorylation MyoIA and MyoID. The anti-phospho antibody did not detect phosphorylated MyoIA or MyoID in crude *Dictyostelium* cell extracts or in immunoprecipitates prepared using the anti-MyoID antibody. Further work is needed to improve the specificity of the anti-phospho MyoID antibody.
Acknowledgments

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Abbreviations

Amp, ampicillin
ATP, adenosine 5'-triphosphate
AX3, Dictyostelium axenic strain 3
Ca²⁺, calcium
cAMP, adenosine 3’5’-cyclic monophosphate
CaM, calmodulin
cAR1, G-protein coupled seven-transmembrane cAMP-receptor
DNA, deoxyribonucleic acid
EA, PakB-null cell
EDTA, ethylene diamine tetraacetic acid
EGTA, ethylene glycol tetraacetic acid
ELC, essential light chain
G418, geneticin
GFT, green fluorescent protein
GST, glutathione S-transferase
His, hexahistidine
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPES</td>
<td>N-(2 hydroxyethyl) piperazine-N’’-2 ethanesulfonic acid</td>
</tr>
<tr>
<td>Kan</td>
<td>kanomycin</td>
</tr>
<tr>
<td>LCBD</td>
<td>light chain binding domain</td>
</tr>
<tr>
<td>MBP</td>
<td>maltose binding protein</td>
</tr>
<tr>
<td>Mg^{2+}</td>
<td>magnesium</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>PAK</td>
<td>p21-activated kinase</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>Pen-Strep</td>
<td>penicillin streptomycin</td>
</tr>
<tr>
<td>RLC</td>
<td>regulatory light chain</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase PCR</td>
</tr>
<tr>
<td>S.cerevisiae</td>
<td><em>Saccharomyces cerevisiae</em></td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SH3</td>
<td>src-homology domain 3</td>
</tr>
<tr>
<td>Stds</td>
<td>standards</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>TBS</td>
<td>tris-(hydroxymethyl) aminomethan buffered saline</td>
</tr>
<tr>
<td>TEDS</td>
<td>threonine, glutamate, aspartate, serine</td>
</tr>
<tr>
<td>TH</td>
<td>tail homology domain</td>
</tr>
<tr>
<td>Tris</td>
<td>tris-(hydroxymethyl) aminomethane</td>
</tr>
<tr>
<td>WB</td>
<td>wash buffer</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

The Life Cycle of *Dictyostelium discoideum*

*Dictyostelium discoideum* was first isolated from the soil of the deciduous forest of North Carolina’s Craggy Mountains by Ken Raper in 1935. *Dictyostelium* is a soil-living amoeba belonging to the phylum Mycetozoa (1). It lives as single cells that divide by binary fissions and feed on bacteria and yeast from deciduous forest soil and decaying leaves. Starvation induces this unicellular amoeba to undergo a developmental life cycle to form a multicellular structure (Figure 1.1) (2). The depleted levels of nutrients stimulate several modifications in the gene expression which allow cells to acquire the ability to produce, secrete, and degrade cAMP. When a small number of unicellular amoeba start to secrete cAMP, neighbouring cells can sense the signal by a cell surface G-protein coupled seven-transmembrane cAMP-receptor (cAR1) that initiates a signalling pathway that stimulates adenylyl cyclase activity and results in the secretion of cAMP (3). The propagating wave of extracellular cAMP results in directed movement along the cAMP gradient. Approximately $10^6$ cells stream towards a chemoattractant center and aggregate to form a mound that later differentiates into a motile slug. Culmination of the developmental cycle occurs when this slug forms a fruiting body, which consists of a stalk that supports a mass of spore cells. The spores disperse into the environment and each spore can germinate to start a new cell under favourable conditions (4).
Figure 1.1. The Development Life Cycle of *Dictyostelium discoideum*. *Dictyostelium* grow as single, vegetative amoebae that undergo a morphogenic cycle to form a mature fruiting body. One important transition between growth and aggregation is mediated by the chemotaxis of cells toward cAMP to generate a multicellular aggregate (2).
Dictyostelium discoideum: A Model System

*Dictyostelium discoideum* has been widely used to study the relationship between the actin cytoskeleton and the production of cellular motility. It provides a simple eukaryotic system, more complex than yeast but much simpler than plants or animals. In *Dictyostelium*, the hereditary information is carried on six chromosomes with sizes ranging from 4-6 Mbp, resulting in a total of about 34 Mbp of DNA. The genome has been fully sequenced and is estimated to contain 10,000 to 13,000 genes (1). To date, over 400 proteins involved in cell motility, signal transduction, and cell differentiation have been identified.

Many cellular processes, such as phagocytosis, cytokinesis, and directed chemotaxis, are found to be more active compared to other model organisms since *Dictyostelium* cells are highly motile throughout their lifetime (4). In addition, *Dictyostelium* are easy to grow in media culture in the laboratory. They grow and divide quickly, with a doubling time of 4 to 12 hrs, on an inexpensive axenic medium at room temperature. *Dictyostelium* genes can also be knocked out in a targeted manner by homologous recombination and the haploid genome allows for easy mutant selection. Several vectors are available for overexpressing proteins in *Dictyostelium* with various tags. Generally, high efficiency transformation is achieved by electroporation. These characteristics make *Dictyostelium* a useful model system to use to understand the molecular interactions underlying differentiation, signal transduction, chemotaxis, and cell motility.
The Myosin Super Family

Myosins are classified as actin-based molecular motors and constitute a diverse superfamily. To date, 18 different classes of myosin are recognized. These myosins share a highly conserved N-terminal ‘head’ or motor domain which serves two main functions: actin filament binding and production of motility by ATP hydrolysis. The motor domain is attached to a ‘neck’ region which serves as a binding site for calmodulin or specialized light chains via a variable number of IQ motifs. Lastly, the structure of the C-terminal tail domain differs widely between different myosins. The tails are thought to play a role in regulating motor activity, in subcellular localization and in cargo binding (5).

*Dictyostelium* expresses thirteen different myosin heavy chain (MHC) genes plus six to eight potential myosin light chain (MLC) genes. These MHC genes can be divided into six classes including one conventional two-headed myosin II, seven single-headed class I myosins (MyoIA-F, MyoIK), two class V myosins (MyoH, MyoJ), and three orphan myosins (MyoG, MyoI and MyoM) that cannot be grouped to any of the already designated classes (6).

Myosin I

The class I myosins were the first discovered unconventional myosin. Myosin I was first isolated and characterized from *Acanthamoeba castellanii* by Pollard and Korn in 1973 (7). Since then myosins I have been found to be present in almost all eukaryotes in many isoforms. In *Dictyostelium*, there are seven myosin I isoforms, MyoIA-F and MyoIK. Class I myosins are single-headed monomeric proteins; that is the tail does not
dimerize or assemble into filaments (8). All class I myosins consist of a conserved N-terminal motor domain that binds actin filaments in an ATP-dependent manner, a neck that binds light chains, and a C-terminal tail domain (9). The N-terminal motor domain possess a surface loop, called the hypertrophic cardiomyopathy loop, which needs to have a negative charge at a particular site in order to form a strong interaction with actin in the absence of ATP (10). This site is known as the TEDS site, due to the fact that the residue found at this position is either a threonine (T), serine (S), aspartic acid (D), or glutamic acid (E) (10). Dictyostelium myosin I have either a threonine or serine at the TEDS site and so need to be phosphorylated in order to be active (Table 1.1). In contrast, most mammalian myosins have either glutamic acid or aspartic acid at the TEDS site, making the motor domain constitutively active (5). Phosphorylation of the TEDS site is needed for myosin I function in vivo. Studies have found that each Dictyostelium myosin I motor domain has different kinetic and functional properties. For example, MyoIB is a low duty ratio motor, meaning MyoIB spends only a short time in the strongly actin-bound states, and it displays the fastest nucleotide binding kinetics (11). Unlike MyoID and MyoIE, MyoIB is not completely inactivated when the TEDS site is dephosphorylated, but can still move actin filaments efficiently (10).

Light Chains Stabilize the Myosin Neck

Over decades, the mechanism by which myosins generate force and motion has been studied intensely throughout eukaryotic cells. Studies have found that the myosin neck functions as a rigid lever arm that swings relative to the head domain to amplify
small conformational changes that occur upon ATP hydrolysis and product release. The 
α-helical neck region contains between one to seven IQ motifs that are about 25 amino 
acids long and conform to the consensus sequence IQxxxRGxxxR (Figure 1.2)(12). IQ 
motifs specifically bind to the light chains, which are thought to play a role in myosin 
regulation; specifically, stabilization of the neck that is needed to produce steps of a 
definite size. The size of the displacement produced by each power stroke is directly 
proportional to the length of the lever arm, which is determined by the number of IQ 
motifs in the neck region (Figure 1.3). Previous in vitro studies have revealed that 
myosins with shorter or less rigid arms move more slowly than wild-type myosins {9} 
Despite of all these findings, it remains unclear whether there are other functions 
associated with the light chains and why different classes of myosin carry varying 
numbers of IQ motifs.

High-resolution crystal structures have been solved for a number of class II 
myosins, which have been crystallized in a variety of nucleotide states. However, the only 
solved crystal structure for a myosin I is of the motor domain of a recombinant 
Dictyostelium MyoIE. Dictyostelium MyoIE shares ~49% sequence similarity with 
Dictyostelium myosin II {Durrwang, 2006 157 /id}. Structural studies show that the 
myosin II lever arm rotates 60–70° from the ATP bound state to the rigor state, whereas, 
the MyoIE lever arm undergoes a rotation of at least 100° during force generation (Figure 
1.4). Thus, for a lever arm of the same length, the MyoIE step size is greater than that of 
myosin II (9,13).
Table 1.1. The TEDS site sequences of the *Dictyostelium* myosins I, MyoA-F and MyoK (targeted amino acids in red).

<table>
<thead>
<tr>
<th></th>
<th>MyoA</th>
<th>MyoB</th>
<th>MyoC</th>
<th>MyoD</th>
<th>MyoE</th>
<th>MyoF</th>
<th>MyoK</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GQGARI<strong>S</strong>TYSVPA</td>
<td>GAGNRR<strong>S</strong>TYNVPQ</td>
<td>HGNQRG<strong>T</strong>QYNVPL</td>
<td>GRSARV<strong>S</strong>TYACPQ</td>
<td>GVGKRC<strong>S</strong>VISVPM</td>
<td>GAGKRQ<strong>S</strong>SIKVLL</td>
<td>SGSARH<strong>T</strong>QYQVPQ</td>
</tr>
</tbody>
</table>
**Figure 1.2. Dictyostelium Myosin I IQ motif sequences.** This table reveals the high sequence variability in the Dictyostelium myosin I IQ motifs. This is in contrast to the conventional myosin II IQ motifs whereby a high degree of conservation is observed.
Figure 1.3. Myosin step size. The size of a step is determined by the length of the rigid lever arm, or light chain binding domain (LCBD). The lever arm is colour-coded red and blue to show states before and after the power stroke, respectively.
Figure 1.4. Docking of class-I and class-II myosin crystal structures onto a model of the actin filament. The α-helical lever arm was modeled onto the crystal structure of the truncated Myo1E head domain to illustrate the power stroke of Myo1E is greater than class-II myosin (13).
Myosin I Tail

The C-terminal myosin I tail comprises of three tail homology (TH) domains: 1) a basic residue-rich membrane binding domain (TH1) that resembles a pleckstrin homology (PH) domain and is required for membrane interactions in vivo, 2) an ATP-independent actin filament binding domain (TH2) with high content of glycine, proline, and alanine/glutamine, and 3) a Src homology 3 (SH3) domain that promotes protein-protein interactions with proline-rich target sequences (PXXP motif) (14). The myosin I isoforms can be categorized into three groups depending on the composition of their tails: the long-tailed, the short-tailed, and the no-tailed (Figure 1.5). The long tailed class I myosins (MyoIB, C, D) contain all three tail homology, TH1, TH2, and SH3, whereas the short tailed class I myosins (MyoIA, E, F) contain only a TH1 domain. MyoIK is the single member that has no tail, although it contains a TH2-like domain as an insert in the motor domain (15).

One of the critical functions of the C-terminal SH3 domain is to couple long-tailed class I myosins to the Arp2/3 complex. The Arp2/3 complex is composed of seven polypeptides and binds to the sides of existing filaments to initiate the growth of a new filament (16). This results in the formation of branched actin filament networks at the leading edges of migrating cells. Yeast class I myosins contain an acidic sequence at the C-terminus of the tail that directly binds to and activates the Arp2/3 complex (17). *Dictyostelium* myosin I isoforms do not have a C-terminal acidic motif, but their SH3 domain interacts with an adaptor protein called CARMIL that binds capping protein and the Arp2/3 complex (Figure 1.6) (16). Interestingly, in *Dictyostelium*, CARMIL, the
Arp2/3 complex, MyoIB and MyoIC all co-localize at actin-rich cellular extensions, including macropinocytic crowns and the leading edge of polarized cells (16).

**In vivo Functions of Class I Myosins**

The myosin I isoforms play distinct, but overlapping, roles in cellular processes such as cell movement, maintenance of cortical tension, phagocytosis, exocytosis, and endocytosis. Several studies have been done using knock-out cells lacking single or multiple myosin I genes to understand the functions of myosin I isoforms (18-22). To date, we do not know the exact functions of myosin I isoforms; however, many studies have observed some of their functions. For example, despite the fact that MyoIA and MyoIB are from two different myosin I subclasses, they play major roles in suppressing lateral pseudopod extension during whole cell motility (23). This suggests that roles of myosin I isoforms do not depend on their subclasses, rather, Ostap et al. predicts that closely related isoforms in *Dictyostelium* can compensate for the loss of an individual myosin I isoform from either class (24). MyoIB was also shown to associate with the plasma membrane during locomotion, the anterior pseudopod, and eupodia (25). MyoIB and MyoIC play significant and probably distinct roles in phagocytosis. Jung et al. found out that the loss of MyoIB is more severe than the absence of MyoIC and MyoID and they predict that cellular concentration of each myosin I isoform is proportional to their contribution to the rate of motility in the cell (8).
Figure 1.5. Domain structures of the Dictyostelium class I myosins. Class I myosins can be divided into three subclasses depending on their tail structures: long-tailed myosins (MyoIB, MyoIC, MyoID), short-tailed myosins (MyoIA, MyoIE, MyoIF) and no-tailed myosin (MyoIK).
Figure 1.6. Myosin I and the Arp2/3 complex. (A) Class I myosins from yeast can interact directly to Arp2/3 complex through its acidic sequence in the tail; (B) however, since long-tail class I myosins from *Dictyostelium* do not have the acidic sequence in their tail, they interact with Arp2/3 complex by binding to an adaptor protein, CARMIL, through their SH3 domain.
Regulatory Mechanisms for Class I Myosins

The detailed mechanisms governing Dictyostelium myosin I regulation are not fully understood. However, there are at least two potential mechanisms that can regulate the activity of the class I myosins: 1) calcium-sensing light chains and 2) heavy chain phosphorylation.

Myosin Light Chains

Myosin light chains belong to the CTER (calmodulin, troponin C, essential light chain (ELC) and regulatory light chain (RLC)) subfamily of EF-hand proteins. Calmodulin is about 16-kDa in size and contains two lobes, which are connected by a flexible linker loop. Each lobe is composed of two EF-hand motifs. A canonical EF-hand motif is approximately 36 amino acids in length and composed of two helices and a 12-residue loop. Calcium chelation occurs through interactions of the metal ion with key residues found within this loop at positions 1, 3, 5, 7, 9 and 12. Specifically, the side chain carboxylate of positions 1, 3, and 5 interact directly with the calcium ion, whereas the residue at position 7 donates a carbonyl oxygen atom from the backbone structure. Strong hydrogen bonds between the residue at position 9 and a water molecule provides another calcium ligand, and the final calcium-chelating ligand occurs by the two side chain carboxylate oxygens of the residue at position 12, which is usually glutamic acid (26). Calmodulin is present in a closed conformation in the absence of calcium, where the hydrophobic residues are buried. In the presence of calcium, the conformation of calmodulin changes causing hydrophobic patches to be exposed, which mediate calcium-calmodulin interactions with a large group of downstream target proteins (27).
**Calcium Regulation of Myosin I by Calmodulin-like Light Chains**

Different myosin I molecules have been shown to associate with different light chains that exhibit varying degrees of calcium sensitivity. A wide diversity of unconventional myosins, including the vertebrate class I myosins, use calmodulin as a light chain. In response to changes in the physiological calcium concentration, the calmodulin light chains can regulate motor activity through the direct binding of calcium. Calcium binding to calmodulin can either inhibit or activate the ATPase activity of vertebrate class I myosins by means of an allosteric mechanism where the calmodulin undergoes a conformational change but remains bound to the neck region. At higher calcium concentrations, calmodulin can dissociate from one or more IQ motifs. This acts as a switch to abolish the motor activity of the class I myosin molecules by weakening the neck region so that it is no longer able to act as a rigid lever arm (28).

In mammals there are a total of eight class I myosin genes which includes six short-tailed and two long-tailed isoform. The first class I myosin identified in vertebrate cells was MyoIa, which is also known as brush-border myosin I. MyoIa is highly enriched in the brush border of intestinal epithelial cells, where it cross-links the actin filament bundle at the core of the microvillus to the plasma membrane (29,30). It binds three to four calmodulin molecules and calcium can regulate its enzymatic activity with different outcomes, depending on the number of calmodulin molecules associated (31,32). The ubiquitously expressed MyoIc has four IQ motifs in its neck region but binds only two to three calmodulins in the absence of calcium and only one calmodulin (to IQ3) in the presence of calcium (33,34). The release of calmodulin in the presence of calcium increases the ATPase activity of MyoIc but completely halts its motor activity (33).
The motor activity of many types of conventional muscle myosin (myosin II) is regulated through phosphorylation of the light chains, but this mode of regulation has not been described for myosin I.

*Dictyostelium* Myosin I Light Chains

Not all of the *Dictyostelium* myosin I light chains have been identified. Recently in our lab, the light chain composition of three class I myosins (MyoIB, MyoIC and MyoID) has been elucidated. Myo1B, Myo1C, and Myo1D all bind to unique light chains (Table 2). Myo1D was the first *Dictyostelium* myosin I to have its light chain characterized (35). Myo1D binds two copies of the unique 16-kDa light chain, MlcD. Although MlcD is a calmodulin-related protein, it does not bind to calcium with high affinity. Interestingly, in contrast to Myo1D, the light chains found to associate with Myo1B and Myo1C (MlcB and MlcC, respectively) are novel in that they are half the size (8-kDa) of a typical myosin light chain (35). That is, they correspond to a single lobe of the 16-kDa calmodulin. MlcB binds calcium with high affinity (36) whereas MlcC has lost the ability to bind calcium (Crawley and Côté, unpublished data).
Table 1.2. The light chain compositions and the domain structures of the *Dictyostelium* class I myosins.

<table>
<thead>
<tr>
<th>Myosin I</th>
<th>Light Chain</th>
<th>Domain Structure</th>
</tr>
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<tbody>
<tr>
<td>MyoA</td>
<td>?</td>
<td></td>
</tr>
<tr>
<td>MyoB</td>
<td>MlcB 8-kDa</td>
<td></td>
</tr>
<tr>
<td>MyoC</td>
<td>MlcC 8-kDa</td>
<td></td>
</tr>
<tr>
<td>MyoD</td>
<td>MlcD 16-kDa</td>
<td></td>
</tr>
<tr>
<td>MyoE</td>
<td>?</td>
<td></td>
</tr>
<tr>
<td>MyoF</td>
<td>?</td>
<td></td>
</tr>
<tr>
<td>MyoK</td>
<td>None</td>
<td>TH2</td>
</tr>
</tbody>
</table>
Two distinct structures for the myosin light chain IQ motif complex

Differences in the IQ motif sequence can result in dramatically different light chain conformation when bound to the motif. Mlc1p is a light chain that binds to IQ motifs of a class V myosin, Myo2p, in *Saccharomyces cerevisiae*. Terrak et al. solved the crystal structure of Mlc1p bound to IQ2 and IQ4 of Myo2p (Figure 1.7) (37). Mlc1p, like calmodulin, is a dumbbell-shaped molecule with two lobes connected by a flexible linker (Figure 1.8). Each lobe contains two EF-hand motifs; however, Mlp1 does not bind calcium. The IQ motif is approximately 25 amino acids long and folds as an uninterrupted seven-turn $\alpha$-helix with a markedly amphiphilic character. The first five turns are facing the hydrophobic core of the C-terminal lobe of Mlc1p and after the fifth turn, the non-polar face flips so that the last two turns are facing the N-terminal lobe. Mlc1p binds to IQ2 of Myo2p in a ‘compact’ conformation where both lobes interact with the IQ motif: the C-lobe interacts with the IQxxxxR portion of the IQ motif and the N-lobe interacts with the GxxxxR part of the IQ motif. On the other hand, Mlc1p binds to IQ4 of Myo2p in an ‘extended’ conformation where only the C-lobe interacts with the IQ motif. The compact conformations depend on the presence of a glycine at position 7 of the IQ motif. In IQ4 Gly7 is replaced with a lysine, which sterically hinders formation of the compact conformation. The loss of the conserved arginine residue at position 11 in IQ4 may also contribute to the extended conformation (37).

In 2006, Houdusse et al. solved a crystal structure of apo-calmodulin bound to the first two IQ motifs of murine myosin V heavy chain to 2.5-Å resolution (38). Myosin V is an unconventional myosin that transports cargo as a monomer in non-muscle cells. The lever arm of myosin V is unusually long and has six IQ motifs, which are stabilized by
the binding of calmodulin or a calmodulin-like light chain. Calmodulin also regulates the activity of myosin V via binding of calcium. The crystal structure of apo-calmodulin bound to the IQ motifs shows that the overall orientation of the IQ motifs and the CaM are antiparallel, with the C-terminal lobe of calmodulin bound to the more N-terminal region of the IQ motifs (Figure. 1.9). Although both calmodulin molecules adopt a similar overall conformation, each calmodulin molecule shows unique interactions with its motif.

Further investigation is needed to explain the specificity of the IQ motif to different calmodulin-like light chains and whether IQ preferences can be regulated in response to cellular signals.
Figure 1.7. Specific sequence variations within the IQ motifs of Myo2p enable two distinct myosin light chain structures. (A) The compact structure of the Mlc1p light chain bound to the IQ2 motif. (B) The extended structure of the Mlc1p light chain bound to the IQ4 motif. The N-lobe, C-lobe and the IQ peptide are represented by the colours blue, red and green, respectively (37).
Figure 1.8. Ribbon diagram of calmodulin. Calmodulin is a dumbbell-shaped molecule consisting of N- and C-terminal lobes, each composed of a pair of EF-hand motifs, connected by a flexible α-helix linker. All myosin light chains belong to the family of calmodulin-related proteins.
Figure 1.9. Structure of the myosin V 2IQ complex. Two Calmodulins (CaM) bound in tandem to the two IQ motifs (gray helix) derived from the sequence adjacent to the motor domain of murine myosin V are shown. Consensus sequence residues (*) of the IQ motif are shown. The orientation of the IQ motifs and calmodulin are antiparallel. A cartoon of the myosin V molecule and the region that was crystallized (red box) (38).
Regulation of *Dictyostelium* Myosin I by Phosphorylation of the Heavy Chain

The p21-activated kinases (PAKs) are a family of serine-threonine protein kinases that are proposed to be key regulators of the actin cytoskeleton and cell motility in organisms ranging from yeast to mammals (15). This family of kinases has been shown to target the TEDS site of the motor domain for activation of myosin I (39). In fact, our lab was the first to demonstrate that a member of the PAK family targets the TEDS site in *Dictyostelium* MyoID (20). PAKs contain two conserved domains: a p21-binding domain (PBD) and a C-terminal Ser/Thr protein kinase catalytic domain (40). PAK kinases exist in an autoinhibited state. There is an autoinhibitory sequence in the PBD that suppresses the activity of the catalytic domain. Binding of GTP-Rac to the PBD releases autoinhibition and allows catalytic activity (Figure 1.10) (8).

There are four isoforms of PAK in *Dictyostelium*: PakA, PakB, PakC and PakD (Figure 1.11). Other than sharing 50-70% sequence identity within the PBD and catalytic domains, PAK isoforms do not exhibit any homology outside of these regions (41). These divergent isoforms are thought to play different cellular roles. PakA is localized to the posterior of migrating cells and its role is not yet confirmed since two groups found two different results with PakA-null mutant cells. One group observed defects in cytokinesis and chemotaxis while others noted no significant behavioral defects (42,43). PakB, originally known as myosin I heavy chain kinase, was the first PAK member to be discovered and characterized. Our lab first isolated PakB as a protein activity capable of phosphorylating and activating MyoID (15). PakB is enriched at the leading edge of migrating cells and in macropinocytic and phagocytic cups (15). These sites are consistent with a role in activating myosin I. PakB is potently activated by human Cdc42 and Rac1 and can bind 7 of the 15 different Racs present in *Dictyostelium*. In addition,
acidic phospholipids can also activate PakB while calcium/calmodulin inhibits activity. PakC is not as well studied as other PAK isoforms; however, it has been found that PakC-null cells exhibit a loss of polarity and produce multiple lateral pseudopodia when placed in a chemoattractant gradient (44,45). PakD has not yet been studied.
Figure 1.10. Schematic activation of PAK from autoinhibited state by GTP-Rac.

PAK has autoinhibitory site at p21-binding domain (PBD), which represses the activity of the catalytic domain. This autoinhibition can be release by binding of Rac-GTP to PBD.
Figure 1.11. Dictyostelium PAK kinase isoforms. Domain drawing of the Dictyostelium PAK kinase isoforms, PakA, PakB, PakC and PakD.
HYPOTHESIS

We hypothesize that differences in their mode of regulation allows Dictyostelium class I myosins to play specific in vivo roles. This differential regulation is achieved through myosin I isoforms: (1) possessing unique light chains (2) having TEDS sites that are phosphorylated by different signal transduction/kinase pathways.

OBJECTIVES

The overall goal is to understand the specific regulatory mechanisms governing the various myosin I isoforms in Dictyostelium and how this differential regulation contributes to the in vivo specificity of each isoform. Our objectives are:

1. Determine the identity of the MyoIA and MyoIE light chains and examine whether or not these myosins are regulated by calcium.
2. Provide evidence that activated RacB leads to downstream phosphorylation of MyoID in vivo.
CHAPTER 2

MATERIALS AND METHODS

Strains and growth conditions

*Dictyostelium* were grown at 21°C in HL5 Axenic Medium containing (per litre):
5 g protease peptone (Oxoid), 5g yeast extract (EMD), 10g glucose, 0.35 g
Na₂HPO₄·7H₂O, and 0.35 g KH₂PO₄. Cells were either grown on 9 cm plastic petri dishes
or in 100 ml conical flasks on a gyratory shaker at 190 rpm. Cells were transformed by
electroporation (Egelhoff et al., 1991). Antibiotics are added to reduce bacterial
contamination: 10,000 units/ml penicillin and 10mg/ml streptomycin (Penicillin-
Streptomycin Solution, Sigma) and 20 μg/ml geneticin (G-418) (Invitrogen). Plasmids
used for transformation were either pTX-FLAG or extrachromosomal vector pDM310.

Cloning of MyoIA-HN and MyoIE-HN

The MyoIA head-neck region (MyoIA-HN; amino acids 1 - 792) and the MyoIE
head-neck region (MyoIE-HN; amino acids 1 - 752) were obtained by RT-PCR using as a
template poly(A) mRNA extracted from growth-phase AX3 cells using the GeneElute
Direct mRNA miniprep kit (Sigma). PCR products were ligated into the pCR2.1 cloning
vector (Invitrogen) and then into the *Dictyostelium* expression vector pTX-FLAG, which
also added an N-terminal FLAG tag to the constructs. Both MyoIA and MyoIE have a
serine at the TEDS site (S332 for MyoIA and S334 for MyoIE). This serine was changed
to glutamic acid in MyoIA-HN and MyoIE-HN using the QuickChange site-directed mutagenesis kit (Stratagene). The fidelity of both constructs were verified by sequencing.

**Expression and purification of MyoIA-HN and MyoIE-HN in Dictyostelium**

*Dictyostelium* Flag-tagged MyoIA-HN and MyoIE-HN were grown in 5 L flasks containing HL5 media. The flasks were incubated on a gyratory shaker (200 rpm, 21°C) until the cells reached a density of 6 x 10^6/ml. Cells were harvested by centrifugation at 4,000 rpm for 20 min in a Beckman J-6 MC centrifuge and washed once in Wash Buffer (2 mM MgCl₂, 20 mM KCl, 10 mM KH₂PO₄, pH 6.8). The wet weight of the resulting cell pellet was measured. The cells were resuspended in 140 ml of Lysis Buffer (50 mM Tris HCl, pH 8.0, 2 mM EDTA, 0.2 mM EGTA, 1 mM dithiothreitol) and centrifuged as before. To induce cell lysis, 50 ml of Lysis Buffer containing 3% Triton-X100, 15 mg/ml RNaseA (Sigma) and 100 units of alkaline phosphatase (Boehringer Mannheim) was added the mixture. The lysate was incubated on ice for 90 min to allow an actomyosin pellet to form. Upon centrifugation at 45,000 rpm for 1 h in a Beckman Ti45 rotor, the MyoIA-HN and MyoIE-HN were recovered in the pellet. The pellet was washed in 100 ml of HKM Low Salt buffer (50 mM HEPES, pH 7.3, 30 mM K acetate, 10 mM MgSO₄, 7 mM β-mercaptoethanol, 5 mM benzamidine, 40 μg/ml PMSF) and centrifuged at 45,000 rpm for 1 h in a Beckman Ti45 rotor. The wash step was repeated three times or until the supernatant looked clear. MyoIA-HN and MyoIE-HN were released into the supernatant by extraction of the pellet with 60 ml HKM High Salt Buffer (HKM Low Salt Buffer + 150 mM KCl) containing 10 mM ATP. After centrifugation at 49,000 rpm for 45
min in a Beckman Ti70 rotor, MyoIA-HN and MyoIE-HN were affinity purified from the supernatant using an anti-FLAG M2 agarose column according to the manufacturer’s instructions (SigmaAldrich). To check the purity of the eluted protein, the eluted samples were run on SDS polyacrylamide gels and the concentration was measured. The pooled fractions were dialysed immediately against Dialysis Buffer (50 mM Tris pH 7.4, 50mM KCl, 1mM DTT, 0.1% Azide, 50% Glycerol). The purified MyoIA-HN and MyoIE-HN could be stored at -80°C for several months without apparent loss of enzymatic activity.

**Expression and purification of calmodulin**

A pET28a vector encoding *Dictyostelium* calmodulin was transformed into *E. coli* strain BL21(DE3). Bacteria were grown in LB to an OD600 of 0.6 and expression was then induced by addition of IPTG to a final concentration of 1 mM. After 8 h at 37°C, cells were harvested, resuspended in ice-cold TBS (50 mM Tris-HCl, pH 7.4, 150 mM NaCl) containing 0.2 mg/mL lysozyme, lysed by sonication and centrifuged at 15,000 x g for 1 h. Calmodulin was recovered in the supernatant and purified over a Ni-NTA His-Bind column (Novagen) followed by chromatography over DEAE anion exchange and Sephacryl S-100 gel filtration columns (Amersham Biosciences). Removal of the His-tag was carried out in buffer containing 500 mM NaCl by addition of 10 units of thrombin (Sigma-Aldrich) per mg of calmodulin. After 16 h at room temperature, samples were passed over a column of benzamidine Sepharose (Pharmacia) to remove thrombin and re-chromatographed over a Sephacryl S-100 column.
Actin-activated Mg\(^{2+}\)-ATPase assays

ATPase activities were measured by the release of \(^{32}\)P\(_i\) from \([\gamma^{\text{32}}\text{P}]\text{ATP}\) as described (46). Actin prepared from rabbit skeletal muscle was added to the assays at a concentration of 30 \(\mu\text{M}\). Reactions were supplemented with calmodulin to ensure that the MyoIA necks were fully occupied by the light chain (47).

Generating \(\alpha\)-MyoID antibody

A MyoID-tail fragment (amino acid 801-1113) was generated by RT-PCR using mRNA isolated from Dictyostelium. The DNA fragment was cloned into the pGEX-4T3 vector to allow expression of the MyoID-tail as a GST-fusion protein. The plasmid was transformed into BL21 (DE3) cells. GST-MyoID-tail was purified from BL21 (DE3) cells by resuspending the frozen pellet in 80 ml of cold 1X PBS, pH 8.0, containing 10 \(\mu\text{l}\) of a protease inhibitor cocktail. The cells were sonicated at 30\% amplitude for 3 min. This step was repeated twice and the cells were then centrifuged at 25,000 rpm for 1 hr. A glutathione column, which was pre-equilibrated in PBS, pH 7.5, was used to affinity purify the GST-MyoID-tail. The protein was eluted using 20 mM glutathione in 1X PBS. The collected fractions were run on an SDS polyacrylamide gel and the concentration of MyoID-tail was measured. Approximately 0.1 mg of GST-MyoID-tail was injected into a rabbit. The polyclonal antibody against MyoID-tail was obtained after 3 months in the serum of the isolated blood. It was tested, using Western blot analysis, against AX3 cell crude extract and it showed strong positive result.
**Generation of a constitutively active RacB isoform in an inducible expression vector**

A constitutively active (CA) form of RacB (V12G RacB) was created using the QuickChange site-directed mutagenesis kit (Stratagene). Both CA-RacB and wild-type RacB were tagged with maltose-binding-protein at the N-terminus domain to allow detection of protein expression. The maltose binding protein coding sequence was obtained from the pMAL-C2X vector (New England Biolabs). *Dictyostelium* cell lines were generated expressing wild-type RacB and CA-RacB under the pDM310 doxycycline-controlled inducible expression system (48). The pDM310 vector, which contains a blasticidin resistance cassette, is repressed in the untreated native cell and induced by conditioned medium in the presence of 10 μg/ml doxycycline.

*Dictyostelium* AX3 and EA cells were cultured in HL5 medium on plastic tissue culture plates at room temperature. Constructs were transformed into cells by electroporation as previously described (49). Cells were selected for growth in HL5 medium containing 10,000 units/ml penicillin and 10 mg/ml streptomycin (Penicillin-Streptomycin Solution, SigmaAldrich) and 20 μg/ml geneticin (G-418) (Invitrogen). After approximately one week, individual *Dictyostelium* colonies were present on each petri dish. Subsequently, cells were plated at limiting dilution in order to produce clonal cell lines. The expression of wild type- and CA-RacB in the AX3 and EA cell lines was confirmed by immunoblot analysis with a monoclonal anti-MBP antibody (New England Biolabs).
**Mass spectrometry analysis**

Myo1A and Myo1E head-neck constructs purified from *Dictyostelium* were separated on an 15% SDS polyacrylamide. Following staining with Coomassie Brilliant Blue, the 17 kDa light chain band was excised from the gel and digested with trypsin and chymotrypsin. Proteolytic digests were analyzed by tandem mass spectrometry (MS/MS) using a Waters Q-TOF Premier mass spectrometer at the Mass Spectrometry Facility in the Department of Chemistry, University of Alberta. MS/MS data was used to identify proteins by an MS/MS Ion Search of the Mascot database.

**Immunoblot analysis**

Samples were mixed with 5X SDS sample buffer, heated at 100 °C for two minutes and subjected to electrophoresis on an SDS polyacrylamide gel. Proteins were then transferred to an Immobilon-P membrane (Millipore) for 1 hour by electrophoresis at 100V. The membrane was incubated overnight at 4 °C with Tween-TBS buffer (20 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 0.02% (v/v) Tween-20) in 5% milk. A 1:10,000 dilution of the monoclonal anti-maltose binding protein antibody (New England Biolabs) in 5% milk solution was applied to the membrane for one hour with shaking at room temperature. The membrane was washed extensively with 1.5% Triton X-100 in PBS. Subsequently, 1:10,000 dilution of the anti-mouse IgG conjugate (Bio-Rad) was applied to the membrane for 1 hour at room temperature. Blots were visualized using an enhanced chemiluminesence system (PerkinElmer) followed by exposure to Kodak X-Omat LS film.
**Immunofluorescence**

For immunofluorescence studies, cells were seeded on glass cover slips coated with 0.1% gelatin for 1 hr at 37 °C. The cover slips were placed in a 24-well tissue culture dishes and cells were grown to near confluence. Doxycycline (10 mg/ml) was added and cells were incubated for 0, 30, 50, 70 and 120 min before being fixed with 95% methanol at -20°C for 10 min. Cells were washed once with PBS, then with 70% ethanol for 10 min at room temperature and then twice with PBS for 15 min. Cells were incubated with 20% normal goat serum in PBS to block non-specific binding of antibodies. Filamentous actin was stained by incubating cells with tetramethylrhodamine isothiocyanate (TRITC)-phalloidin (Sigma-Aldrich) diluted 1:250 in PBS for 1 hr at room temperature in the dark. Following two washes with PBS, coverslips were mounted to slides using an anti-fade mounting solution (Dako) and examined using a Zeiss Axiovert S100 inverted fluorescence microscope equipped with a 40X oil immersion lens. Images were captured using a high sensitivity Cooke SensiCam and SlideBook software (Intelligent Imaging Innovations Inc.).
CHAPTER 3

RESULTS

Part 1.

Previously in our lab, we identified the light chain composition of three Dictyostelium myosin I isoforms (MyoIB, MyoIC and MyoID) (36). Endogenous MyoIB and MyoID were purified using conventional isolation techniques whereas MyoIC was purified from recombinant expression of Flag-tagged head-neck constructs (Crawley and Côté, unpublished data). MyoIB, MyoIC and MyoID are all long-tailed class I myosins. The goal of this study was to identify the light chain composition for two short-tailed class I myosin isoforms (MyoIA and MyoIE) in Dictyostelium and determine whether their enzymatic activities are regulated by calcium.

Purification and SDS-PAGE analysis of MyoIA-HN and MyoIE-HN

Flag-tagged MyoIA and MyoIE head-neck constructs (Myo1A-HN, amino acids 1–792 and MyoIE-HN, amino acids 1–752) were expressed in Dictyostelium cells. Cells were lysed in Triton X-100 to prepare actin cytoskeletons. Depletion of cellular ATP was used to recruit most of the endogenous and recombinant myosins into a rigor-like complex with the actin cytoskeleton. Following a series of washes with a low salt buffers, a high salt buffer containing 10 mM ATP was added to extract the myosin protein from the actin cytoskeleton. The Flag-tagged MyoIA-HN and MyoIE-HN were then affinity
purified using an anti-Flag resin. The purified proteins were analyzed by SDS polyacrylamide gel electrophoresis. The Coomassie blue-stained gel showed that the purification of MyoIA-HN and MyoIE-HN resulted in the co-purification of a 17-kDa protein, which was in the range corresponding to that of known myosin light chains (Figure 3.1). These results raised the possibility that MyoIA-HN and MyoIE-HN contains a 17-kDa light chain.

In a control experiment, a MyoIA construct containing only the head region, without the neck, (Myo1A-H) was expressed in Dictyostelium cells and purified. When MyoIA-H was examined on a Coomassie blue-stained SDS gel, the 17 kDa protein could not be detected (Figure 3.2). This result provides evidence that the 17-kDa protein specifically associates with the IQ motifs in the neck region of MyoIA.

**Identification of a putative MyoIA light chain and MyoIE light chain**

To determine whether the putative light chain associated with MyoIA-HN and MyoIE-HN is a calcium-binding protein, we added calcium or EGTA and ran it on an SDS gel (Figure 3.3). The Coomassie blue-stained gels showed that the 17-kDa protein exhibited a calcium-dependent mobility shift. The same samples were used for a Western blot analysis using a monoclonal Dictyostelium anti-calmodulin antibody (15). The antibody successfully reacted with the MyoIA and MyoIE light chains. Finally, the identity of the 17-kDa light chains was confirmed to be Dictyostelium calmodulin by mass spectrometry (Figure 3.4). These studies show that MyoIA and MyoIE have calmodulin as their light chains.
Figure 3.1. SDS-PAGE analysis of MyoIA-HN and MyoIE-HN purified from *Dictyostelium* The Coomassie blue-stained SDS gels show (A) the purified MyoIA-HN electrophoresed on a 12% gel, (B) the purified MyoIE-HN construct electrophoresed on a 12% gel. Bands corresponding to the myosin I head-neck (HN) and putative light chains are indicated. MyoIA-HN and MyoIE-HN co-purified with a prominent 17-kDa light chain.
Figure 3.2. SDS-PAGE analysis of MyoIA head from *Dictyostelium*. The Coomassie blue-stained SDS gel shows purified MyoIA-H electrophoresed on a 12% gel. No protein is associated with MyoIA-H, providing evidence that the 17-kDa protein that co-purifies with MyoIA-HN is specifically associated with the IQ motifs in the Myo1A neck region.
Figure 3.3. MyoIA-HN co-purified with a 17-kDa light chain. (A) The 17-kDa light chain exhibits a mobility shift in the presence of calcium. (B) A monoclonal anti-

*Dictyostelium* calmodulin antibody reacts with the 17-kDa MyoIA light chain.
Figure 3.4. Mass spectrometry data. The sequence of Dictyostelium calmodulin is shown and the peptides that were identified by mass spectrometry from MyoIA (red) and MyoIE (blue) are underlined.
Co-sedimentation of MyoIA-HN and MyoIE-HN

Studies on mammalian class I myosins have found that the presence of calcium promotes dissociation of one or more of the calmodulin light chains (31). An actin co-sedimentation assay was used to examine the degree of calcium sensitivity of the MyoIA and MyoIE calmodulin light chains. MyoIA-HN and MyoIE-HN were incubated with filamentous actin and various concentration of calcium (25 μM – 250 μM) in the absence of ATP. Following an ultracentrifugation step to pellet the filamentous actin, the pellet was analyzed by SDS-PAGE. The Coomassie blue-stained SDS gel showed that the calmodulin light chains do not dissociate from MyoIA-HN (Figure 3.5, panel A) and MyoIE-HN (Figure 3.5, panel B) in the presence of calcium.

Actin-activated Mg$^{2+}$-ATPase activity of MyoIA-HN and MyoIE-HN

A number of studies have demonstrated that the actin-activated Mg$^{2+}$-ATPase activities of mammalian class I myosins that have calmodulin light chains are regulated by calcium (46). To examine the possibility that the motor activities of MyoIA and MyoIE are regulated by calcium, actin-activated Mg$^{2+}$-ATPase activity assays were performed.

The recombinant MyoIA-HN and MyoIE-HN constructs used in these studies contained a point mutation that changed the serine residue at the TEDS site (S332 for MyoIA and S334 for MyoIE) to glutamic acid. The negatively charged glutamic acid at the TEDS site makes the MyoIA-HN and MyoIE-HNs constitutively active so that they
do not need to be phosphorylated. As expected, MyoIA-HN (Figure 3.6, panel A) and MyoIE-HN (Figure 3.6, panel B) have very low Mg$^{2+}$-ATPase activities in the absence of F-actin. When actin filaments were added, there was 5- and 6-fold increase in the Mg$^{2+}$-ATPase activity of MyoIA-HN and MyoIE-HN, respectively. The addition of calcium did not have any effect on the actin-activated Mg$^{2+}$-ATPase activities of MyoIA-HN (Figure 3.6, panel A) or MyoIE-HN (Figure 3.6, panel B).
Figure 3.5. MyoIA co-sedimentation assay. The Coomassie blue-stained SDS gels show that the calmodulin (CaM) light chains do not dissociate from MyoIA-HN. The graph shows the densitometry.
Figure 3.6. MyoIE co-sedimentation assay. The Coomassie blue-stained SDS gels show that the calmodulin (CaM) light chains do not dissociate from MyoIE-HN. The graph shows the densitometry.
Figure 3.7. Actin-activated Mg$^{2+}$-ATPase activity assay (A) MyoIA-HN and (B) MyoIE-HN were assayed in the presence and the absence of 30 µM rabbit skeletal muscle actin, presence of calmodulin, and in the presence and absence of 250 µM calcium. Calcium did not have any effect on the MyoIA-HN and MyoIE-HN Mg$^{2+}$-ATPase activities.
**Table 3.1.** The light chain compositions and the domain structures of the *Dictyostelium* class I myosins.

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<th>Light Chain</th>
<th>Domain Structure</th>
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Part 2.

Myosin I Heavy Chain Phosphorylation by p21-activated kinase (PAK)

To determine the \textit{in vitro} myosin I substrate phosphorylation specificity of the \textit{Dictyostelium} PAK family, we tested GST-tagged kinase domains of PakA and PakC purified from \textit{E. coli} with the isolated head domains of MyoIA, MyoIB, MyoIC, MyoID and MyoIE expressed and purified from \textit{Dictyostelium}. PakA and PakC were used to phosphorylate each myosin head domain construct \textit{in vitro}. It was found that both PakA and PakC can phosphorylate MyoIA, MyoID, and MyoIE. However, MyoIB and MyoIC were poor substrates for PAKs (Figure 3.7, panel A), possibly indicating that these myosins are regulated by a different signal transduction/kinase pathway. We were unable to obtain the PakB kinase catalytic domain from expression in \textit{E. coli}.

Generation of reagents to examine the effect of \textit{Dictyostelium} RacB on the phosphorylation of MyoID

Previous work has shown that PakB binds strongly \textit{in vitro} to \textit{Dictyostelium} RacB (15). Studies were carried out to assess whether the expression of a constitutively active RacB isoform stimulated MyoID phosphorylation \textit{in vivo}. To achieve this, we made three components, including the inducible wild-type (WT)- and constitutively active (CA)-RacB cell lines, a polyclonal antibody against MyoID for pull-down assays and a phosphospecific antibody against the MyoID TEDS site for Western blot analysis. We also assessed the level of MyoID phosphorylation in a knock-out cell line which lacks PakB (EA cell). A non-induced cell line was used as a control in both cases.
We generated constructs which express WT-RacB and CA-RacB in a doxycycline-controlled inducible vector (pDM310) system (48). This inducible discoidin promoter is repressed in the untreated native cell and induced by conditioned medium in the presence of doxycycline (10 µg/ml). Maltose-binding-protein (MBP) was fused to the N-terminus of WT-RacB and CA-RacB to allow detection of protein expression. The vectors were transformed into AX3 and EA cells. In the absence of doxycycline, the MBP-CA-RacB was undetectable. However, after cells were grown in the presence of 10 µg/ml doxycycline, the expression of MBP-CA-RacB was successfully detected using an anti-MBP antibody (Figure 3.8).

To generate an antibody toward MyoID for immunoprecipitation, the DNA encoding a MyoID-tail fragment (amino acids 801-1113) was obtained by PCR (Figure 3.9, panel A). The DNA fragment was cloned into the pGEX-4T3 vector to produce a MyoID-tail GST-fusion protein (Figure 3.9, panel B). The GST-MyoID-tail fusion protein was successfully purified from E. coli (Figure 3.10, panel A) and was used to immunize a rabbit. The anti-MyoID-tail rabbit antibody was tested against AX3 crude cell extract and successfully detected the 125-kDa MyoID heavy chain (Figure 3.10, panel C). A protein of ~40-kDa also reacted with the anti-MyoID antibody and may represent a proteolytic breakdown product of MyoID.

An antibody against a phosphorylated peptide corresponding to the MyoID TEDS site was previously generated by Dr. Linda Shen in Dr. Alan Mak’s lab (Queen’s University). The antibody specifically detects the phosphorylated forms of purified MyoIA and MyoID head constructs, but does not detect phosphorylated MyoIB, MyoIC.
and MyoIE head constructs (Figure 3.11, Crawley and Côté, unpublished data). No cross reaction with the unphosphorylated purified myosin I proteins was observed.

After successfully preparing these essential components, the plasmids encoding WT-RacB and CA-RacB were transfected into Dictyostelium AX3 and EA cell lines. Gene expression was activated by adding 10 μg/ml doxycycline to the media. After a day of treatment, cells were lysed and total MyoID was immunoprecipitated using the polyclonal anti-MyoID antibody; however, when the immunoprecipitate was blotted against anti-MyoID or anti-phosphospecific antibody, it did not detect a band corresponding to MyoID (data not shown). The anti-MyoID can detect MyoID from the crude extract strongly but somehow it was not useful for immunoprecipitation.

**RacB induced changes in the F-actin cytoskeleton**

Studies were carried out to examine whether the amount of filamentous actin is elevated in cells that over express RacB. AX3 and EA cells expressing CA-RacB were collected 0, 30, 50, 70 and 120 min after induction with doxycycline (Figure 3.12 and Figure 3.13). The cells were fixed and stained with TRITC-phalloidin and confocal z sections were acquired. The exposure time was consistent throughout the experiment; therefore, graphs were generated by quantifying the amount of filamentous actin by measuring the intensity of red in TRITC-phalloidin. Expression of CA-RacB in AX3 cells resulted in almost a 3-fold increase in filamentous actin from 0 min to 120 min (Figure 3.12, graph). In contrast, CA-RacB in EA cells showed less than a 1.5-fold increase in F-actin intensity (Figure 3.13, graph).
It was also observed that AX3 cells adhered to the surface of the plate stronger than EA cells. However, following induction of CA-RacB expression by addition of doxycycline, the EA cells adhered more strongly than the AX3 cells (data not shown).
Figure 3.8. Specificity of PakA and PakC phosphorylation of MyoIA - MyoIE and MyoIA - MyoIE TEDS site sequences (A) The autoradiogram shows that bacterially-expressed Dictostelium PakA and PakC catalytic domains phosphorylate MyoIA, MyoID and MyoIE (Crawley and Cote, unpublished data). (B) The TEDS site sequences of the Dictostelium myosins I, MyoIA-E (targeted amino acids in red).
Figure 3.9. *Doxycycline-inducible RacB expression system.* Whole cell extracts from *Dictyostelium* expressing pDM310-MBP-CA-RacB in WT AX3 cells and in PakB-null cells (EA) were subjected to immunoblot analysis using anti-MBP antibody. Only the cells that were induced with doxycycline were successfully detected. The successful pDM310-MBP-protein, which was previously made for other experiments, was used as a control.
Figure 3.10. Cloning and purification of a MyoID-tail fragment. A) RT-PCR was used to obtain DNA encoding the MyoID tail from Dictyostelium mRNA. B) To verify the cloning of MyoID-tail into the pGEX-4T3 vector, restriction enzymes were used to excise the insert encoding the MyoID-tail fragment.
Figure 3.1. Generation of a rabbit polyclonal antibody to the MyoID tail. (A) The Coomassie blue-stained SDS gel shows the GST-MyoID-tail purified from *E. coli*. (B) The Coomassie blue-stained SDS gel shows a whole cell extract of *Dictyostelium* AX3 cells. (C) The *Dictyostelium* whole cell extract was subjected to immunoblot analysis using the rabbit polyclonal anti-MyoID antibody. A band of the correct size was observed at around 125-kDa for the MyoID heavy chain.
Figure 3.12. Specificity of the phosphospecific antibody against MyoID. A phosphospecific antibody generated against a peptide corresponding to the MyoID TEDS site detects phosphorylated MyoIA and MyoID, but not MyoIB, MyoIC and MyoIE.
Figure 3.13. Effect of CA-RacB expression on the amount of filamentous actin in AX3 cells. Cells were grown in the absence of doxycycline and collected after 0, 30, 50, 70 and 120 min after addition of doxycycline. The cells were fixed and stained with TRITC-phalloidin to visualize actin filaments and confocal z sections were acquired. The graph shows the intensity of actin staining at each time point. The average and standard deviation were obtained by measuring the fluorescence intensity for 10 cells at each time point.
Figure 3.14. Effect of CA-RacB expression on the amount of filamentous actin in EA cells. Cells were grown in the absence of doxycycline and collected 0, 30, 50, 70 and 120 min after addition of doxycycline. The cells were fixed and stained with TRITC-phalloidin to visualize actin filaments and confocal z sections were acquired. The graph shows the intensity of actin staining at each time point. The average and standard deviation were obtained by measuring the fluorescence intensity for 10 cells at each time point.
CHAPTER 4

DISCUSSION

A primary focus of the current thesis is to identify the light chains of the short-tailed *Dictyostelium* class I myosins, MyoIA and MyoIE, and to determine whether their enzymatic activities are regulated by calcium. The second part is to whether a constitutively active form of RacB leads to the TEDS site phosphorylation of MyoID.

**Calmodulin light chain**

Calmodulin-like light chains bind to sequences in the myosin neck region known as IQ motifs that contain the generalized consensus sequence IQxxxRGxxxR, where x is any amino acid (31). The tandemly repeated IQ motifs of unconventional myosins can vary in length from 22 to 25 residues and the number of bound calmodulin-like light chains can vary as well. Therefore, calmodulin may adopt different orientations and conformation with respect to each other. The vertebrate myosins I employ the ubiquitous Ca$^{2+}$-sensor protein calmodulin as a light chain, and their motor activity can be modulated by the binding of calcium to the calmodulin light chains. *Dictyostelium* calmodulin is 152 amino acids long and also involved in regulating actin-myosin interactions. It was first discovered in 1980 based on its head stability and its activation of brain cyclic nucleotide phosphodiesterase in the presence of calcium (32). Subsequently, a single gene (calA) was shown to code for *Dictyostelium* calmodulin producing an mRNA that is expressed
constitutively throughout growth and development. It possesses four high-affinity EF-hand binding sites, which is similar in structure and function to vertebrate calmodulin. The *Dictyostelium* calmodulin and vertebrate calmodulin shares 88.7% sequence similarity (50). *Dictyostelium* calmodulin localizes primarily to membrane of the osmoregulatory system, for example, contractile vacuoles, probably due to its Ca\(^{2+}\)-independent association via IQ motifs in the neck domain of an unconventional myosin on the surface of the vacuole (47).

In the present study, we used co-immunoprecipitation and SDS-PAGE analysis to show that MyoIA and MyoIE, both short-tailed *Dictyostelium* myosin I isoforms, are associated with a 17-kDa light chain. The 17-kDa light chain exhibited a mobility shift in the presence of calcium which is typical of calmodulin. The light chains also reacted with an anti-calmodulin antibody and were confirmed to be calmodulin by mass spectrometry. It has been found that the motile activities of several mammalian myosin I isoforms associated with calmodulin light chains are modulated by physiological levels of calcium. For example, mammalian MyoIc has three calmodulin light chain binding IQ motifs and is bound by two to three calmodulins in the absence of calcium (33). Elevated calcium concentrations induce one or more calmodulin to dissociate from MyoIc which results in increased MgATPase activity but halts *in vitro* motility (51). Rat MyoIB has six IQ motifs in the neck region and its actin-activated ATPase activity is increased 4-fold in the presence of calcium (52). One calmodulin dissociates from rat Myo1b in the presence of calcium. In our study, co-sedimentation assays showed that the calmodulin light chains did not dissociate from *Dictyostelium* MyoIA and MyoIE in the presence of calcium.
The activity of the myosin motor domain can be regulated by a variety of mechanisms including: calcium binding to the regulatory light chain, the phosphorylation of the regulatory light chain, or the phosphorylation of a site in the motor domain of the heavy chain (28). The vertebrate smooth muscle myosin and Dictyostelium class II myosin require phosphorylation on its regulatory light chain by the calcium–calmodulin-dependent enzyme, myosin light chain kinase (8). On the other hand, in Dictyostelium, it has been found that heavy chain phosphorylation plays important roles in the activation of Dictyostelium myosin I. Specifically, Dictyostelium class I myosin requires phosphorylation of a serine or threonine residue located on the heavy chain in a surface loop of the motor domain (TEDS site) that is thought to interact with actin.

The essential role of light chains is to stabilize the α-helical myosin neck, which can functions as a rigid lever arm to amplify the conformational changes in the motor domain. Therefore, one possible explanation for why calmodulin light chains do not dissociate from MyoIA and MyoIE in the presence of calcium is that they may be necessary to make the neck region more rigid for the efficient powerstroke.

**Regulatory roles of calmodulin light chain**

Light chains can be critical sites of regulation of myosin activity through phosphorylation or by the direct binding of calcium (18). In vertebrate cells, MyoIa is the first class I myosin that was identified and it binds to 3-4 calmodulin molecules and as a result calcium can regulate its enzymatic activity. Elevated level of calcium promotes an increase of actin-activated Mg²⁺-ATPase activity of rat MyoIb and MyoIc but inhibits the
actin-activated Mg\(^{2+}\)-ATPase activity of rat MyoId and MyoIe (51,52,52). Thus, we performed actin-activated Mg\(^{2+}\)-ATPase activity assay to see whether calcium has any effect on MyoIA and MyoIE enzymatic activities. From our studies, no evidence for the direct regulation by calcium was found. Although these studies were performed with truncated version of MyoIA and MyoIE lacking the tail, removal of the tail was shown not to effect regulation of rat MyoIb and MyoIc by calcium. However, this result does not rule out the possibility that calcium affects the mechanical properties of MyoIA and MyoIE or that it plays a role in motility or in localization in the cell. Further studies on the full-length and truncated form of MyoIA and MyoIE will be required using motility assays to see whether the binding of calcium to the calmodulin light chains has any effect on their motor activities.

It is interesting that the long tailed myosin I isoforms (MyoIB, MyoIC, and MyoID) have unique light chains, whereas the short tailed myosin I isoforms (MyoIA and MyoIE) have calmodulin as their light chains. The length and sequence of the IQ motifs varies between the different class I myosins. The difference in IQ motifs may explain the different specificities exhibited by the IQ motifs for the various myosin light chains. It has been shown that sequence variations in the IQ motif affect the mode of light chain binding. Terrak et al. found that the C-terminal half of the IQ motif (GxxxxR) interacts with the N-lobe of the light chain and the N-terminal half of the IQ motif (IQxxxR) interacts with the C-lobe of the light chain (37). When both lobes interact with the IQ motif they form a ‘compact conformation’. This compact conformation requires the presence of a glycine at position 7 and an arginine or possibly a lysine at position 11 in IQ motif consensus sequence. The ‘extended conformation’ is formed when only the C-lobe
of the light chain interacts with the IQ motif. This is due to the presence of a lysine, or any other bulky side chains, at position 7 that sterically hinders the interaction of the N-lobe of the light chains with the IQ motif.

Variations in IQ motifs among the different myosins may also explain the difference in calcium effects on ATPase activities. Previous studies have found that calcium sometimes stimulates (53), inhibit (54) or have essentially no effect (31) on actin-activated Mg\textsuperscript{2+}-ATPase activities of myosins I. There is a possibility that sequences outside the minimal IQ motif may be involved in the specific recognition of light chains. The reason why myosin I isoforms have different light chains and what determines the specificity of the IQ motif for a particular light chain is at present not well understood. Further studies on the myosin I IQ motifs will be required to identify the features that confer specificity for its light chain.

**Downstream pathway of constitutively active RacB**

Rho family of GTPases serve as a molecular switches that regulate many essential cellular processes, including actin dynamics, gene transcription, cell-cycle progression and cell adhesion (41). They are one of the important regulators of F-actin polymerization in cell movement (23,44). So far, six classes of Rho family proteins have been identified in humans, Rho, Rac, Cdc42, Rnd, RhoD, and TTF (55). In *Dictyostelium*, 14 Rho-related proteins have been identified and are named Rac1a, Rac1b, Rac1c and RacA–J (56). Only a few of the *Dictyostelium* Racs have been characterized. *Dictyostelium* RacC overexpression leads to the formation of an unusual actin-containing protrusion at the cell
surface and a three-fold increase in phagocytosis while macropinocytosis rates were significantly reduced (41). RacE is essential for cytokinesis but is not involved in processes such as phagocytosis, chemotaxis and development (57). RacF1 localizes to early phagosomes and macropinosomes but inactivation of the RacF1 gene does not impair endocytosis and other actin-dependent processes, probably due to the presence of a closely related RacF2 (56). Overexpression of the constitutively active form of Rac1 generated lamellopodia, inhibited macropinocytosis, and resulted in defects in growth and cytokinesis. Overexpression of the dominant negative form of Rac1 protein induced only modest effects on cell morphology and inhibited chemotactic cell movement (58). Lee et al. proposed that the RacB is normally involved in the control of cortical actin polymerization (59). They also showed that the dominant negative form of RacB similar morphology to wild-type RacB; however, cells expressing constitutively active RacB tend to detached from the substrate and formed many surface protrusions and stopped growing (59). Together, these data indicate that Dictyostelium Rac1 proteins play a role in regulation of actin cytoskeleton and actin related activities comparable to that observed for mammalian cells.

Members of the p21-activated kinase (PAK) family, which are activated by Rac, are key regulators of the actin cytoskeleton and cell motility in organisms ranging from yeast to mammals (60). Dictyostelium PakB, previously termed myosin I heavy chain kinase, is known to be activated by RacB isoforms (15). PakB was identified on the basis of its ability to phosphorylate and activate MyoID (46). PakB is enriched at the leading edge of migrating cells, which is consistent with a role in the regulation of class I myosins (8). Previously in our lab, we have found that the constitutively active form of PakB
increases the rates of pinocytosis and phagocytosis, which are two myosin I-dependent processes, and disrupts cytokinesis. Constitutively active PakB is concentrated at the posterior of migrating cells, suggesting a model in which the N-terminal domain of activated PakB attaches tightly to cortical actin filaments that flow to the rear of the cell (15). In contrast, PakB-null (EA) cells exhibited no significant defects in a variety of motile processes known to be dependent on myosin I, including cell migration, phagocytosis and pinocytosis (15). One possible explanation might be that the loss of PakB is compensated for by other Dictyostelium protein kinases, such as PakA or PakC.

We wanted to explore whether expression of CA-RacB increased the levels of MyoID phosphorylation in vivo. We expressed CA-RacB in a doxycycline-controlled inducible vector (pDM310) system in Dictyostelium but could not detect any phosphorylated MyoID when the anti-phospho-MyoID antibody was used to probe Dictyostelium crude cell extracts. The anti-phospho-MyoID antibody specifically reacted with purified phosphorylated MyoIA and MyoID, suggesting that the levels of phosphorylated MyoID are too low to be detected or that the anti-phospho-MyoID antibody displays a low affinity for phosphorylated MyoID. Therefore, we tried to enrich for MyoID by immunoprecipitation with a rabbit antibody against MyoID but this was not successful. Although the anti-MyoID blot detected a very strong intensive band when used for an immunoblot with a crude cell extract, it did not work well for the pull-down experiments. For further study, a stronger anti-phospho-MyoID antibody should be generated that can be detect phosphorylated MyoID in a crude cell extract without having to perform immunoprecipitation.
**Phenotypic consequences of activated RacB**

In previous studies have found that PakB is enriched at the leading edge of the migrating cell and PakB is known to phosphorylate MyoID isoforms (15). Class I myosin plays a role in actin filament assembly by coupling with Arp2/3 complex via adopter protein called CARMIL. Therefore, we wanted to compare the actin intensity between pDM310 CA-RacB in AX3 cells and pDM310 CA-RacB in PakB-null (EA) cells to see whether the absence of PakB plays a role in the actin filamentsation.

Cells overexpressing RacB in AX3 and EA cells under an inducible promoter were transformed. Changes in cell morphology in the constitutively active RacB were observed: 1) AX3 cells adhere to the surface of the plate stronger than EA cells, 2) induced CA-RacB EA cells adhere stronger than CA-RacB AX3 cells. This corresponds to the previous studies that the CA-RacB blocks cell growth and/or the cell cycle rather just cytokinesis (55). The previous studies, done by Lee et al., have established the morphology of WT- and CA-RacB (59). They observed that the CA-RacB stopped dividing for at least 36 hours, which correspond to our observation throughout the experiments. Also, they have found that the CA-RacB cells became detached from the surface and formed many spherical surface protrusions. They could be actin polymerization-dependent pseudopods, water-filled membrane blebs, or a deformation of the actin cortex and plasma membrane; however, it is most likely due to an increase in the amount of F-actin polymerization in cells.

When CA-RacB was induced with doxycycline, the cells were taken and fixed in a time course: 0, 30, 50, 70 and 120 min, then stained with TRITC-phalloidin, which stains
F-actin. Expression of CA-RacB in AX3 cells increased almost a 3-fold in filamentous actin from 0 min to 120 min, whereas, CA-RacB in EA cells showed only a 1.5-fold increase in F-actin intensity within 120 min. From these results, we conclude that the absence of PakB has an effect on actin filament assembly; however, further studies should be done to confirm these results. For example, another cell line should be generated with dominant negative form of RacB to compare with the constitutively active form to make sure that the effect is indeed due to RacB. Also, the same experiment should be performed using knock-out of another or multiple Pak isoforms, such as PakC since RacB also activates PakC, cell lines to observe the difference results to observe the specificity of these Pak isoforms.

Future perspectives could focus on screening the Dictyostelium kinome to search for other kinases capable of phosphorylating and activating the myosin I isoforms (61). Through identifying the upstream signalling pathways of individual myosin I isoforms that lead to their activation, we will gain a better understanding of the specific roles each myosin has been adapted to. In addition, it would be interesting to investigate whether there are subpopulations that exist of individual myosin I isoforms that possess differential light chain compositions that may specify unique attributes such as specific cellular localizations.
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