Actin Activation of Myosin Heavy Chain Kinase A in *Dictyostelium*: A BIOCHEMICAL MECHANISM FOR THE SPATIAL REGULATION OF MYOSIN II FILAMENT DISASSEMBLY*

By: Thomas T. Egelhoff, Daniel Croft, and Paul A. Steimle

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Abstract:

Studies in *Dictyostelium discoideum* have established that the cycle of myosin II bipolar filament assembly and disassembly controls the temporal and spatial localization of myosin II during critical cellular processes, such as cytokinesis and cell locomotion. Myosin heavy chain kinase A (MHCK A) is a key enzyme regulating myosin II filament disassembly through myosin heavy chain phosphorylation in *Dictyostelium*. Under various cellular conditions, MHCK A is recruited to actin-rich cortical sites and is preferentially enriched at sites of pseudopod formation, and thus MHCK A is proposed to play a role in regulating localized disassembly of myosin II filaments in the cell. MHCK A possesses an aminoterminal coiled-coil domain that participates in the oligomerization, cellular localization, and actin binding activities of the kinase. In the current study, we show that the interaction between the coiled-coil domain of MHCK A and filamentous actin leads to an ~40-fold increase in the initial rate of kinase catalytic activity. Actin-mediated activation of MHCK A involves increased rates of kinase autophosphorylation and requires the presence of the coiled-coil domain. Structure-function analyses revealed that the coiled-coil domain alone binds to actin filaments (apparent K_D = 0.9 μM) and thus mediates the direct interaction with F-actin required for MHCK A activation. Collectively, these results indicate that MHCK A recruitment to actin-rich sites could lead to localized activation of the kinase via direct interaction with actin filaments, and thus this mode of kinase regulation may represent an important mechanism by which the cell achieves localized disassembly of myosin II filaments required for specific changes in cell shape.

Article:

Myosin II plays an important role in a wide variety of contractile events in eukaryotic cells by supporting ATP-dependent movement of actin filaments. Studies in the amoeboid protozoan *Dictyostelium discoideum* have revealed that complex cellular processes such as cytokinesis, cellular migration, maintenance of cell cortical tension, cell surface receptor capping, and multicellular development depend on the proper function and regulation of myosin II (1). The diversity of these myosin-dependent processes indicates that cells must be able to undergo highly localized reorganization of their myosin II assemblies in response to signals initiating specific contractile processes (1, 2). For example, myosin II bipolar filaments accumulate transiently at the cleavage furrow of dividing cells where they contribute to the separation of the two daughter cells via localized contraction of actin filaments (3–5). In another context, myosin II filaments localize to the posterior of migrating cells where they contribute to directed cell movement by facilitating retraction of the cell rear and suppressing lateral pseudopod formation (3, 6–8).

In *Dictyostelium*, as well as in mammalian non-muscle cells (2), myosin II exists in a dynamic equilibrium between a cytoplasmic pool of monomers and a cytoskeleton-associated assembly of bipolar filaments (9, 10). Bipolar filament assembly appears to be required for the translocation of myosin II to the cortical actin

cytoskeleton (11) and thus may be a critical process regulating myosin localization and activity in response to specific signals such as exposure of cells to chemoattractants (10, 12–15). Biochemical and cellular studies have shown that the ability of *Dictyostelium* myosin II to assemble into filaments is inhibited by phosphorylation of three threonine residues located near the carboxyl terminus of the myosin II heavy chain (MHC)[1] (11, 16–18). These phosphorylation sites were initially recognized as target sites for the enzyme myosin heavy chain kinase A (MHCK A) (17, 18); studies exploring the regulation of this kinase are the central focus of this paper.

Myosin II filament assembly in *Dictyostelium* is regulated via MHC phosphorylation through the combined activities of at least one MHC phosphatase (19, 20) and a group of MHC kinases (9, 21, 22). MHCK A was the first of the heavy chain kinases to be identified and purified (23), and cellular and biochemical studies of MHCK A demonstrated that the activity of this kinase drives myosin filament disassembly both *in vitro* and *in vivo* by phosphorylating the regulatory threonine sites of MHC (23, 24). MHCK A exhibits a molecular mass of 130 kDa under denaturing conditions but appears to be oligomeric under native conditions with a molecular mass of >700 kDa (23). Detailed studies of MHCK A catalytic activity revealed that unlike most serine/threonine protein kinases, MHCK A preferentially phosphorylates threonine residues (25). Other studies have shown that MHCK A autophosphorylation, which leads to a 50-fold increase in kinase activity, is stimulated not only by its substrate, myosin II, but also by acidic phospholipids (26, 27).

Cloning and subsequent sequence analysis of the MHCK A gene revealed a novel modular organization to the protein (28). The amino-terminal domain of MHCK A is predicted to have significant α-helical coiled-coil structure and has been shown biochemically to mediate MHCK A oligomerization (29). The central catalytic region of MHCK A is the prototype for a novel family of protein kinases, referred to as "α-kinases," which do not exhibit any apparent sequence similarity to any of the conventional protein kinase families (28, 30, 31). Representatives of this kinase family have been identified in diverse groups of organisms including several kinases in *Dictyostelium* (9, 31), as well as others in mammalian systems that include eukaryotic elongation factor-2 kinases and the Channel kinase TRPM7/ChaK1 (30). At its carboxyl terminus, MHCK A possesses a domain containing a 7-fold WD repeat motif (32) that facilitates phosphorylation of myosin heavy chain by physically targeting MHCK A directly to myosin II filaments (33).

The studies described above established that MHCK A, which is expressed during both vegetative growth and multi-cellular development (24), can drive myosin II filament disassembly *in vitro* and *in vivo* by phosphorylating specific regulatory sites in MHC. Thus, the cellular distribution of MHCK A activity is likely to have an important effect in regulating the spatial localization of myosin II during contraction-dependent processes such as cytokinesis and chemotaxis. During the aggregation stage of development, *Dictyostelium* cells respond to a gradient of the chemoattractant cAMP by becoming highly polarized and then forming cellular streams that move toward the source of chemoattractant (34). Localization studies in chemotaxing cells revealed that MHCK A is recruited to the actin-rich cell cortex upon chemoattractant stimulation and is preferentially recruited to sites of pseudopod formation at the cell anterior (35). Most interestingly, the translocation activity of MHCK A requires cellular actin filaments but does not depend on the presence of myosin II in the cell. In the context of cytokinesis, MHCK A is enriched at the actin-rich polar regions of the dividing cell (21, 22). The accumulation of MHCK A at actin-rich cell extensions, coupled with reduced levels of myosin II at such sites (9, 36), suggests a model in which myosin II filament assembly is prevented as a result of MHCK A recruitment to such sites. Consequently, myosin II-based cross-linking of actin filaments at regions forming cellular extensions may be relaxed; in turn, this may promote the assembly and reorganization of the actin cytoskeleton necessary for the formation of pseudopodial structures (35, 37–41).

Insight on the structural factors contributing to the dynamic changes in MHCK A localization has come from *in vivo* localization studies of GFP-tagged truncations of MHCK A in live *Dictyostelium* cells (42). This study revealed that the amino-terminal coiled-coil region of MHCK A is both necessary and sufficient for mediating cAMP-stimulated translocation of the kinase to the cell cortex. Accompanying experiments demonstrated that like full-length MHCK A, the coiled-coil domain alone exhibits persistent localization to both anterior and lateral cell protrusions and that cortical localization of the truncation requires the presence of an intact

filamentous actin cytoskeleton. Parallel actin-binding experiments showed that although full-length MHCK A interacts with actin filaments, a truncation of the kinase lacking the coiled-coil domain was unable to associate with F-actin, suggesting that the determinants for association with F-actin reside in this domain of MHCK A (42).

In summary, previous research has established that cortical recruitment and anterior localization of MHCK A require the presence of actin filaments and are mediated by the coiled-coil domain of the kinase (35, 42). *In vitro* assays have shown that the coiled-coil domain is required for MHCK A binding to actin filaments (35). In the current study, we explore the hypothesis that the interaction between the coiled-coil domain of MHCK A and F-actin has the potential to regulate MHCK A activity, and thus may be a central factor controlling localized disassembly of myosin II filaments in the cell. Studies examining the functional consequence of the interaction between F-actin and MHCK A revealed that the catalytic activity of MHCK A is stimulated dramatically up to 40-fold in the presence of actin filaments. Further studies examining the mechanism of activation revealed that F-actin promotes MHCK A autophosphorylation, which leads to increased initial rates of kinase phosphorylation of substrate. Structure-function analyses revealed that the coiled-coil domain indeed binds to actin filaments and that this interaction is required for F-actin stimulation of MHCK A activity. Taken together, these results indicate that MHCK A localized to actin-rich sites of the cell may become activated upon interaction between the coiled-coil domain of the kinase and actin filaments, ultimately leading to the localized disassembly of myosin II filaments from such sites.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—Amino acid residue numbering in this text refers to the GenBank™ sequence entry for MHCK A (accession number P42527). For kinase assays, both full-length MHCK A and the ΔCoil-MHCK A truncation (lacking residues 1–498) were cloned into plasmids for expression in *Dictyostelium* cells as fusion proteins with amino-terminal His_6 tags. The construction of these recombinant plasmids, as well as the purification of the overexpressed MHCK A (full-length) and ΔCoil-MHCK A proteins, has been described in detail previously (29).

In preparation for actin-binding experiments, MHCK A gene truncations encoding either the coiled-coil domain alone (GST-Coil; amino acids 1–498) or the catalytic domain alone (GST-CAT; amino acids 499–841) were cloned into the pGEX-2T vector (Amersham Biosciences) for expression as fusion proteins with amino-terminal glutathione *S*-transferase (GST) tags. Preparation and cloning of the sequence encoding the catalytic domain of MHCK A have been described elsewhere (33). Cloning of the coiled-coil domain for this study involved using standard DNA manipulations and materials. Briefly, the coiled-coil domain truncation was amplified by PCR methodology using TaqDNA polymerase, in-frame primers containing BamHI sites, and the cloned full-length MHCK A gene as template (28). PCR-amplified coiled-coil domain was ligated directly into the pDrive PCR cloning vector (Qiagen Inc., Valencia, CA); recombinant vector (pDrive-Coil) was then introduced into *Escherichia coli* (DH5α) bacteria using standard transformation procedures.

Bacterial clones containing pDrive-Coil recombinant plasmid were selected via ampicillin resistance and blue-white screening on LB agar plates supplemented with 150 μg/ml ampicillin, 80 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; Promega Corp.), and 50 μg/ml isopropyl β-D-thiogalactopyranoside (IPTG; Promega Corp.). Clones forming white colonies were transferred to liquid culture (LB medium supplemented with 150 μg/ml ampicillin; Sigma), grown overnight at 37 °C with shaking (200 rpm), and then harvested via centrifugation for purification of pDrive-Coil plasmid using isolation protocols provided by Qiagen. The presence of the coiled-coil domain insert was determined via BamHI restriction enzyme digest of the recombinant plasmid. The fidelity of the cloned coiled-coil domain sequence was verified by DNA sequencing (Sequencing Core Facility, University of North Carolina, Chapel Hill) and comparison with the known sequence of MHCK A (GenBank™ accession number U16856).

The coiled-coil domain insert was subcloned into the bacterial expression vector pGEX-2T by first digesting the pDrive-Coil plasmid (10 μg) with BamHI (30 units) for 2 h at 37 °C to excise the coiled-coil domain insert and then gel-purifying the excised fragment using the QIAEX II gel extraction protocol (Qiagen). The purified

coiled-coil domain fragment was then ligated into BamHI-linearized pGEX-2T vector. *E. coli* DH5α cells were transformed with an aliquot of the ligation reaction, and clones containing recombinant plasmid were selected via ampicillin-resistant growth on LB-agar plates supplemented with 150 μg/ml ampicillin (37 °C overnight incubation). Several ampicillin-resistant colonies were then transferred to liquid culture (LB medium supplemented with 150 μg/ml ampicillin), grown overnight at 37 °C with shaking (200 rpm), and then harvested for plasmid isolation as described above. The presence and orientation of the coiled-coil domain fragment in the pGEX-2T vector were determined via restriction enzyme mapping and DNA sequencing; recombinant pGEX-2T plasmid containing the coiled-coil domain fragment in the correct orientation was purified on a large scale for introduction into bacterial expression hosts.

Protein Expression and Purification—Full-length MHCK A and ΔCoil-MHCK A used in kinase assays were overexpressed in *Dictyostelium* cells and purified to homogeneity as described previously (29). For actin-binding experiments, GST-tagged fusion proteins containing either the coiled-coil domain (GST-Coil) or the catalytic domain (GST-CAT) of MHCK A were expressed in bacteria and then purified to homogeneity via affinity chromatography. The expression, purification, and characterization of the GST-CAT fusion protein have been described previously (33). For the expression and purification of the GST-Coil fusion protein, *E. coli* cells (BL21(DE3)-Rosetta®; Novagen Corp.) were transformed with recombinant pGEX-2T vector containing the coiled-coil domain insert. The transformed bacterial cells were then grown overnight (37 °C with shaking) in 20 ml of LB medium containing ampicillin (150 μg/ml). After incubation, the overnight culture was transferred to a flask containing 1 liter of LB plus ampicillin (150 μg/ml), and cells were grown to log phase ($A_{600} \approx 0.6$) at 37 °C with shaking. At this time, the log phase culture was cooled to room temperature (~22 °C). Cells were then induced to express GST-Coil by adding IPTG (1 mM final concentration) and then incubating for at least 3 h at room temperature with shaking. The IPTG-induced cells were then harvested by centrifugation (10,000 × *g* for 20 min); the supernatant was discarded, and the pellet frozen at -80 °C. The frozen pellet was either processed immediately for extraction of the fusion protein or stored frozen for up to a week before processing.

Frozen pellets of induced cells were thawed and resuspended in 5 ml of phosphate-buffered saline (PBS, 10 mM sodium phosphate, 100 mM NaCl), containing 1 mM EDTA, 2 mM dithiothreitol, 1× protease inhibitor mixture (PIC; Roche Diagnostics). The cell suspension was incubated with 5 mg/ml lysozyme for 15 min on ice and then sonicated by subjecting the cells to five pulses (Branson sonicator; 10 s at setting 3) with 30-s incubations on ice between each pulse to prevent sample heating. The resulting sonicate was then centrifuged for 40 min at 21,000 × *g* (4 °C), and the supernatant was collected for further purification of the GST-Coil fusion protein.

All purification steps described here were performed at 4 °C unless indicated otherwise. GST-Coil was purified to homogeneity via affinity chromatography by incubating the soluble fraction of the sonicate with glutathione-Sepharose beads (GST·Bind™ Resin; Novagen) for 1–3 h with rotation. Unbound material was removed by washing the beads with at least 5 column volumes of PBS. The bead-bound GST-Coil fusion protein was then eluted by incubating the beads with 10 mM soluble glutathione (in PBS containing PIC) for 12 h with rotation. Released GST-Coil fusion protein was collected into a single tube and then dialyzed overnight against 2 liters of 20 mM Tris, pH 7.5, in 10% glycerol. The dialyzed GST-Coil protein was analyzed by both SDS-PAGE/Coomassie Blue staining and immunoblotting with anti-GST antibody to assess the purity and stability of the eluted protein. Protein quantification involved SDS-PAGE/Coomassie Blue staining of samples of purified GST-Coil or GST-CAT and known amounts of pure bovine serum albumin standards and then comparing relative band intensities between these samples (33).

Actin Polymerization—In preparation for kinase assays containing filamentous actin, as well as for actin co-sedimentation experiments, lyophilized rabbit skeletal muscle actin (Cytoskeleton, Inc., Denver, CO) was resuspended in 200 μl of distilled H_2O to give a 119 μM stock solution of actin in 5 mM Tris-HCl, pH 8.0, 0.2 mM $CaCl_2$, 0.2 mM ATP, and 0.5 mM DTT (G-Buffer). Actin filament assembly was induced by diluting the actin stock in a final volume of 50 μl containing 5 mM Tris, pH 8.0, 0.2 mM $CaCl_2$, 0.5 mM DTT, 100 mM

KCl, 2 mM $MgCl_2$, and 1 mM ATP (F-buffer); this solution of actin (50 μM) was incubated for 60 min at 25 °C to allow polymerization to occur. If necessary, the solution of polymerized actin was diluted in F-buffer to give a 100× concentration that was then added to the kinase or co-sedimentation assay mixes.

Kinase Assays—Purified MHCK A and the ΔCoil truncation of MHCK A were assayed for their ability to phosphorylate a peptide substrate in the presence or absence of actin filaments by using essentially the same kinase assay described previously (27, 33). Unless indicated otherwise, the kinase activity was assessed in the presence or absence of F-actin (3–5 μM) in a reaction mix (50–100 μl) containing 10 mM TES, pH 7.0, 7 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.62 mM [γ-^{32}P]ATP (100–250 Ci/mol), and 50 μM MH-1 peptide substrate (RKKFGESEKTKTKEFL-amide). The MH-1 peptide has been described previously (25) and contains the mapped MHCK A target site in the myosin II tail (underlined in peptide sequence above). As a note, the addition of F-actin to the reaction mix results in the addition of KCl to 10 mM and an increase in the ATP concentration from 0.5 to 0.62 mM. Negative control reactions (lacking F-actin) were supplemented with KCl, as well as with additional $MgCl_2$ and ATP, to give the same final concentrations found in F-actin-containing kinase reactions. Kinase reactions were initiated by the addition of either full-length MHCK A or ΔCoil-MHCK A at 20 nM; reaction mixes were incubated at 25 °C for various lengths of times as indicated for each experiment. Where indicated, 5 μM latrunculin A (Sigma) was added to kinase reactions from a 10× concentrated stock solution of the reagent dissolved in dimethyl sulfoxide (Me_2SO); an equivalent volume of Me_2SO vehicle was added to negative control reactions lacking latrunculin A.

Unless indicated otherwise, neither full-length nor ΔCoil MHCK A were allowed to autophosphorylate prior to initiating each kinase reaction. At each time point after initiation of the kinase reaction, 10-μl aliquots of the reaction mix were removed, and the reaction was stopped by immediately blotting the aliquot onto P-81 filter paper (Whatman, Inc.); the filters were then processed for scintillation counting to determine the extent of ^{32}P incorporation into MH-1 peptide (43). Phosphorylation values were subtracted from those obtained from negative control assays in which the peptide substrate was omitted.

Autophosphorylation assays were performed essentially as described previously (33) by incubating either full-length MHCK A or its ΔCoil truncation (both at 100 nM) in the same kinase reaction mix described above but lacking peptide substrate and containing 5 μM filamentous actin where indicated. At the appropriate time points, 20-μl aliquots of the autophosphorylation reaction mix were transferred to 10 μl of 100 °C Laemmli sample buffer (five times) containing 5 mM EDTA and then incubated at 100 °C for 5 min. The level of kinase autophosphorylation was assessed by subjecting the aliquots to SDS-PAGE and autoradiography followed by excision of Coomassie-stained bands for quantification of phosphorylation via scintillation counting.

Actin Co-sedimentation Assays—Binding to actin filaments was assessed via a standard co-sedimentation assay, which has been described in detail previously (42). Briefly, F-actin co-sedimentation assays were performed by incubating GST fusion proteins (0.15 μM) with F-actin (5 μM)ina60-μl reaction mix containing 5 mM Tris, pH 8.0, 0.2 mM $CaCl_2$, 0.5 mM DTT, 100 mM KCl, 2 mM $MgCl_2$, 1 mM ATP, and 1× PIC. The reaction mix was incubated for 15 min at 25 °C to allow for interaction between the fusion protein and F-actin. Negative control reactions containing all the components of the assay except F-actin were performed in parallel. After incubation, the co-sedimentation reactions were centrifuged at 100,000 × *g* for 20 min at 4 °C to sediment actin filaments. The resulting supernatant fractions were transferred to fresh tubes, and pellet fractions were resuspended in 60 μl of cosedimentation reaction buffer (described above). Equal volumes of the pellet and supernatant fractions were resolved by SDS-PAGE followed by Coomassie staining to detect the actin and GST fusion proteins present in each fraction. The level of fusion protein co-sedimentation with F-actin was quantified via scanning densitometry of stained gels.

RESULTS

Previous studies have revealed that MHCK A binds directly to actin filaments *in vitro* and that a truncation of MHCK A lacking the putative actin-binding domain does not exhibit chemoattractant-stimulated translocation to the actin-rich cell cortex (42). Taken together, these results indicate that a direct interaction with F-actin may

play a prominent role in regulating MHCK A cellular localization and potentially affecting the activity of the kinase. In the current study, we explored the functional consequence of the interaction between F-actin and MHCK A by comparing MHCK A phosphorylation of a specific peptide substrate (MH-1) in the presence and absence of F-actin. Results from these assays revealed that the initial rate of MHCK A activity is induced ~40-fold in the presence of F-actin (Fig. 1*A*, 30-s time point); this induction is reflected in the elimination of the initial lag in activity observed with the unstimulated kinase. However, with time the level of induction decreases, with only a 2.5-fold activation of MHCK A observed 4 min after the start of the kinase reaction (Fig. 1*A*).

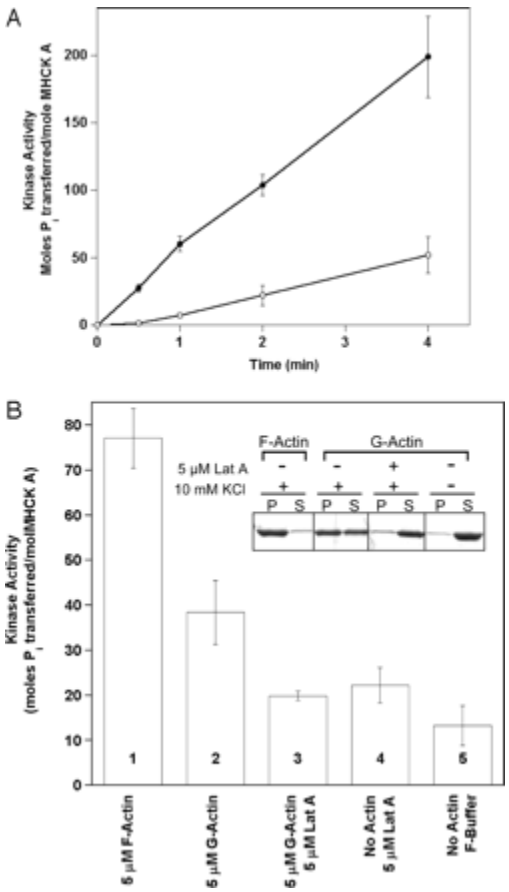

Fig. 1.
MHCK A-mediated phosphorylation of MH-1 peptide is activated in the presence of polymerized actin. *A*, the phosphorylation of MH-1 peptide substrate by full-length MHCK A (20 nm) was assessed over time in the presence (•) or absence (○) of 5 μm F-actin as described under "Experimental Procedures." *B, bar graph* of MHCK A phosphorylation of MH-1 in a kinase reaction (1 min) performed in the presence or absence of 5 μm actin under various assay conditions (as described under "Experimental Procedures"). The *inset* is a Coomassie Blue-stained SDS-polyacrylamide gel showing the distribution of actin in pellet (*P*) and supernatant (*S*) fractions resulting from high speed centrifugation (100,000 × *g* for 20 min) of kinase reactions containing 5 μm actin. The presence or absence of 10 mm KCl, 5 μm latrunculin A, or vehicle (Me_2SO) in each reaction mix is indicated above the corresponding pellet and supernatant fractions. The pellet and supernatant fractions *bracketed* by *F-actin* were derived from a kinase reaction in which polymerized actin was added to the mix. Those fractions *bracketed* by *G-actin* originated from kinase reactions in which non-polymerized actin was added to each mix. The *plotted points* and *bars* depicted in both *A* and *B*, respectively, represent the mean values from at least three separate experiments. The *vertical lines* indicate the S.E.

The specificity of the F-actin-stimulated increase in MHCK A activity was tested by performing control assays in which either monomeric actin (G-actin) or no actin was included in the reaction mix. Unless indicated otherwise, all kinase reactions were supplemented with KCl, $MgCl_2$, and ATP to give the same final concentrations of these components present in F-actin-containing kinase reactions (10 mM KCl, 7 mM $MgCl_2$, and 0.62 μM ATP, respectively). Under these conditions, a significant (but reduced) increase in MHCK A activity was observed in the presence of G-actin (Fig. 1*B*; compare *bars 1, 2,* and *5*). Further exploration revealed that the kinase reaction conditions described above result in a significant level (40–60%) of G-actin

polymerization (Fig. 1*B, inset*); this is consistent with the well documented polymerization of actin at higher concentrations of KCl and $MgCl_2$ (44).

The apparent G-actin-induced increase in MHCK A activity was abolished upon the addition of the actin-depolymerizing drug, latrunculin A (Lat A), to the reaction mix (Fig. 1*B, bar 3*). This corresponds with the elimination of actin filaments in the reaction mix (Fig. 1*B, inset*) and suggests that it is the presence of actin filaments (not G-actin) that activates MHCK A. Control experiments demonstrated that Lat A does not affect MHCK A activity directly because the kinase exhibits base-line levels of activity in the presence of Lat A alone (Fig. 1*B, bar 4*). Moreover, Me_2SO (the vehicle for Lat A) does not interfere with MHCK A activity because the presence of Me_2SO alone does not eliminate the increase in kinase activity observed under conditions in which G-actin polymerization takes place (data not shown). Taken together, these data demonstrate that the activation of MHCK A observed in initial assays (Fig. 1*A*) is indeed due to the presence of actin filaments and is not a consequence of a non-specific enhancement of kinase activity in the presence of actin protein. As a note, MHCK A phosphorylation of actin was not detectable (data not shown).

The structural requirements of F-actin-specific activation of MHCK A were explored in kinase assays (±F-actin) of an MHCK A truncation lacking the coiled-coil domain (Δ-Coil-MHCK A); the coiled-coil domain has been shown previously to be required for both cellular translocation and actin binding by MHCK A (42). Results from these assays revealed that the Δ-Coil truncation, unlike full-length MHCK A, is not activated by F-actin (Fig. 2). This is not due to a deficiency in the kinase activity of the Δ-Coil truncation of MHCK A because the ability of the truncation to phosphorylate MH-1 substrate has been shown to equal that exhibited by full-length MHCK A (29). The rising activity slope of the Δ-Coil construct in both conditions in Fig. 2 reflects the activation of this construct by autophosphorylation, as was observed in earlier analyses where the MHCK A coil domain was removed (45). Taken together, these studies indicate not only that a direct interaction between the coiled-coil domain and actin is necessary for F-actin-mediated activation of MHCK A but also that the stimulatory effect of autophosphorylation is retained even in the absence of the coil domain.

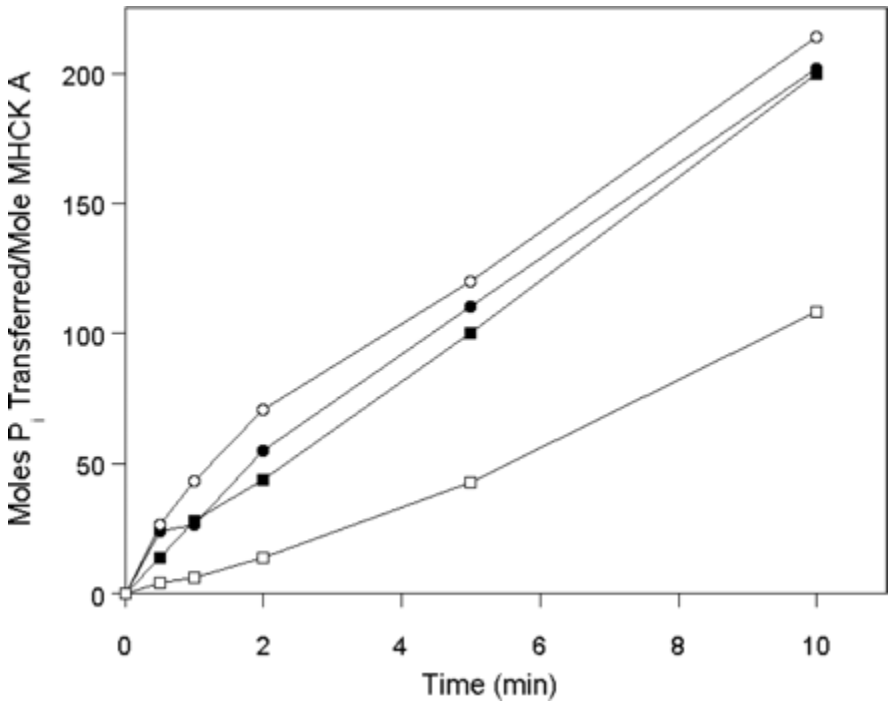

Fig. 3.
Autophosphorylated MHCK-A is not activated in the presence of F-actin. MHCK A autophosphorylation was performed as described under "Experimental Procedures" by incubating MHCK A in the kinase reaction mix for 20 min prior to adding MH-1 peptide substrate to start the kinase reaction. Actin-induced activation of full-length MHCK A that was either pre-autophosphorylated before initiation of the assay (•) or not preautophosphorylated before the initiation of the assay (▪) was compared over time in the presence of 5 μm F-actin. For comparison, the activity of autophosphorylated (○) and non-phosphorylated MHCK A (□) was assessed over time in reactions lacking of F-actin.

To the test the possibility that F-actin facilitates autophosphorylation of MHCK A, the phosphorylation of purified MHCK A (100 nM) was assessed over time in the presence and absence of 5 μM F-actin. Results from these assays revealed that the initial rate of MHCK A autophosphorylation in the presence of F-actin is significantly higher than that in reactions lacking F-actin (Fig. 4, *closed* and *open squares*). As suggested by the graph in Fig. 4, the level of MHCK A autophosphorylation in the presence or absence of F-actin eventually reaches the same maximum (~10 mol P_i/mol MHCK A). In contrast to full-length MHCK A, the autophosphorylation activity of the Δ-Coil truncation of MHCK A is not affected by F-actin (Fig. 4, *closed* and *open circles*). We also show, as has been reported previously (29), that in the absence of F-actin the Δ-Coil truncation of MHCK A displays the same rate of autophosphorylation as full-length MHCK A under the same conditions (Fig. 4), indicating that the Δ-Coil truncation does not possess an autophosphorylation defect.

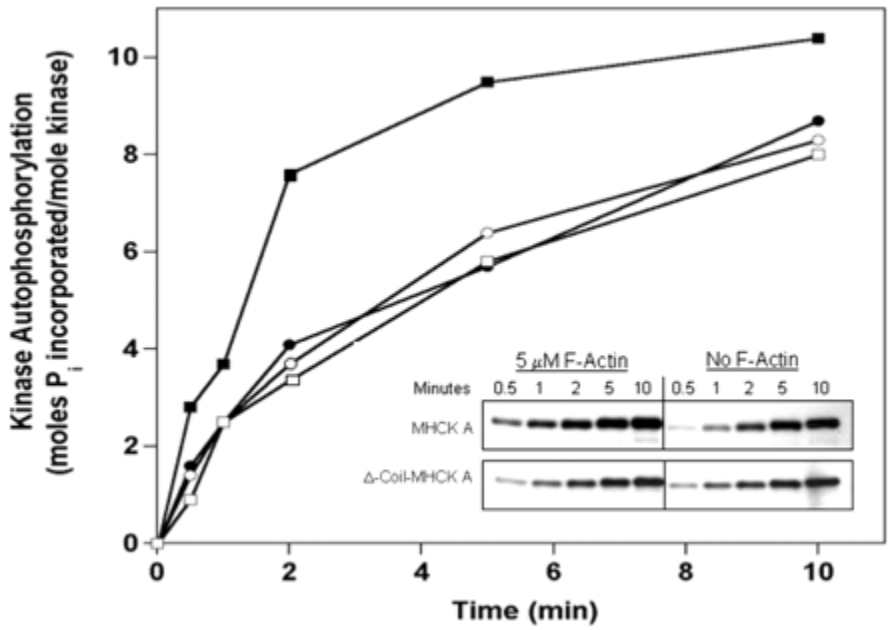

Fig. 4.
F-actin stimulates MHCK A autophosphorylation via a mechanism requiring the coiled-coil domain of the kinase. Autophosphorylation reactions for both full-length MHCK A and the ΔCoil truncation were performed at 100 nm concentration of each protein in the presence or absence of 5 μm F-actin. The level of autophosphorylation was determined by subjecting aliquots of autophosphorylation reactions at specific time points to SDS-PAGE, Coomassie Blue staining, and then scintillation counting of excised kinase bands. The level of full-length MHCK A autophosphorylation in the presence (▪) or absence (□) of F-actin was plotted over time. Likewise, ΔCoil-MHCK A autophosphorylation in reactions containing (•) or lacking (○) F-actin were plotted. Visual analysis of MHCK A and ΔCoil autophosphorylation (see *inset*) was achieved via autoradiography of dried, Coomassie-stained SDS-polyacrylamide gels of autophosphorylation time points.

The results discussed thus far suggest a model in which F-actin stimulates autophosphorylation of MHCK A upon binding directly to the coiled-coil domain of the kinase; these events ultimately lead to MHCK A activation. To test the ability of the coiled-coil domain to interact with F-actin, a purified GST-tagged fusion protein containing the entire coiled-coil domain of MHCK A (amino acids 1–498; GST-Coil) was assayed for co-sedimentation with actin filaments. Analysis of the resulting pellets and supernatants by SDS-PAGE and Coomassie Blue staining revealed that the GST-Coil fusion protein indeed co-sediments with actin filaments; for comparison, the coiled-coil protein alone (without actin filaments) was subjected to the same assay and was found to remain in the supernatant (Fig. 5*A*). GST-Coil binding to actin filaments is concentration-dependent with maximal binding achieved at about 0.15 mol of GST-Coil/mol of actin (Fig. 5*B*). The apparent K_D for GST-Coil interaction with F-actin is ~0.9 μM GST-Coil. Actin co-sedimentation experiments in which GST-Coil is replaced with either GST-CAT fusion protein or GST alone (without a fusion partner) were performed as negative controls for non-specific co-sedimentation (Fig. 5*A*). The GST-CAT fusion protein contains only the catalytic domain of MHCK A and is not predicted to possess actin binding activity (42). A comparison of the level of actin binding by each fusion protein showed that the percent of total fusion protein (the amount in both the pellet and supernatant fractions) co-sedimenting with F-actin was significantly lower for GST (1.4% ± 0.93,

n = 3) and GST-CAT (4.4% ± 1.7, *n* = 3) when compared with that exhibited by GST-Coil (42.4% ± 3.9, *n* = 3) under the same conditions. The results described above, together with previous data (8), demonstrate that the coiled-coil domain of MHCK A is both necessary and sufficient for MHCK A binding to actin filaments.

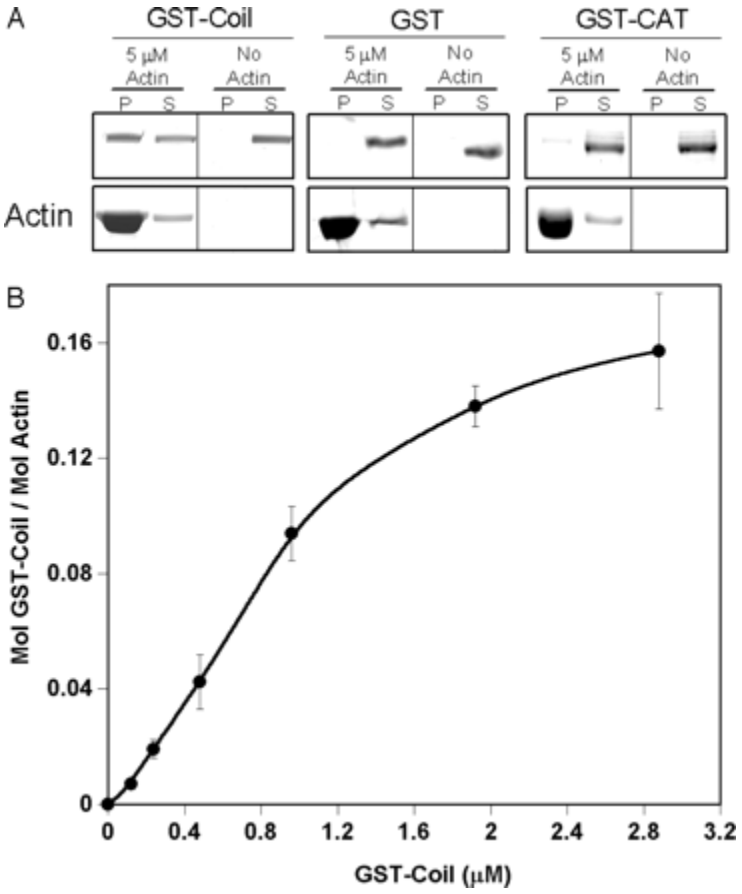

Fig. 5.
The coiled-coil domain of MHCK A binds directly to filamentous actin. *A*, GST-tagged proteins (0.15 μm) were incubated (25 °C for 15 min) with 5 μm rabbit skeletal muscle F-actin and then subjected to ultracentrifugation as described under "Experimental Procedures." The resulting pellet (*P*) and supernatant (*S*) fractions were subjected to SDS-PAGE and Coomassie Blue staining to visualize co-sedimentation of the fusion proteins with F-actin in the pellet fraction. *B*, GST-Coiled-coil protein (0.5–3 μm) was incubated with 3 μm F-actin, and the amount of fusion protein co-sedimenting with actin filaments was quantified via scanning densitometry of stained gels. The amount of GST-Coiled-coil protein bound (mol/mol actin) is plotted against the concentration of fusion protein.

DISCUSSION

The results presented here establish that MHCK A, a key regulator of myosin II filament assembly in *D. discoideum*, is activated dramatically upon direct interaction with actin filaments. Previous studies (1) have demonstrated that MHCK A catalyzes the disassembly of myosin II bipolar filaments into monomers by phosphorylating specific sites in the tail region of myosin heavy chain. The resulting myosin II monomers are unable to contract opposing actin filaments; thus the cellular localization of MHCK A is liable to have an important impact on the filamentous state, activity, and distribution of myosin II in the cell. Cell-based studies have shown that MHCK A undergoes regulated recruitment to actin-rich cortical sites in the cell and exhibits preferential enrichment at anterior and lateral regions of protrusive activity (35, 42). In contrast, myosin II is essentially absent from the leading edge of a migrating cell but is highly enriched at the cell rear (1). Likewise, MHCK A and myosin II exhibit opposite distributions during cytokinesis with MHCK A enriched at the polar ends of the dividing cell and myosin II localized to the cleavage furrow (21, 22). The results presented in this paper extend our current understanding of how myosin II filament assembly can be regulated in a non-muscle cell context and provide an important addition to a working model in which disassembly of myosin II filaments can be confined to specific regions of the cell by targeting not only the localization but also the activation of MHCK A to specific actin-rich sites in the cell.

Initial experiments described in this paper tested the hypothesis that if MHCK A interacts directly with actin filaments (42), then F-actin may play a role in regulating the activity of the kinase. Results from these experiments demonstrated that the initial rate of MHCK A phosphorylation of a peptide substrate is activated ~40-fold in the presence of filamentous actin (Fig. 1); monomeric actin has no effect on the activity of the kinase. We also show that F-actin-mediated stimulation of MHCK A activity is dependent on a direct interaction between the two proteins because the coiled-coil domain, which is required for binding to actin filaments (42), is also required for kinase activation by actin (Fig. 2). Further exploration of the interaction between MHCK A and F-actin revealed that the coiled-coil domain alone possesses the structural determinants for binding to actin filaments (Fig. 5) and thus demonstrates that this domain is both necessary and sufficient for MHCK A binding to F-actin.

MHCK A binding to F-actin leads to a striking increase in the rate of kinase autophosphorylation (Fig. 4). As reported previously (27), autophosphorylation is a central mechanism by which MHCK A is activated. Most interestingly, MHCK A that is already in the autophosphorylated state is not activated further in the presence of F-actin (Fig. 3), suggesting that stimulation of MHCK A is achieved via increased autophosphorylation and does not involve an additional activation mechanism. Studies by Côté *et al.* (45) suggest that the region of MHCK A (amino acids 499–551) linking the coiled-coil domain to the catalytic domain functions as an autoinhibitory sequence that can be released upon autophosphorylation. In contrast, the coiled-coil domain does not appear to be involved directly in autoinhibition of kinase activity because the Δ-coil truncation, whereas not stimulated by F-actin, is able to autophosphorylate and become activated upon autophosphorylation (Fig. 2) (29, 45).

Collectively, these data suggest a model in which coiled-coil domain binding to F-actin triggers a conformational change in MHCK A that releases the autoinhibitory constraints placed on the catalytic domain of the kinase (Fig. 6). In this model, actin-induced changes in MHCK A structure would favor phosphorylation of regulatory sites in the autoinhibitory domain; this autophosphorylation leads to the release of autoinhibition and stabilization of the active conformation of the kinase catalytic core. Alternatively, it is possible that actin-driven activation of MHCK A may in fact be independent of autophosphorylation. In this scenario, the transition to the activated state is actually achieved upon interaction with F-actin, and the increased rate of MHCK A autophosphorylation is simply a consequence of the overall increase in kinase activity resulting from actin-induced release of autoinhibition. In this case, a version of MHCK A unable to autophosphorylate would still be activated in the presence of F-actin. Future studies exploring MHCK A regulation will focus on identifying the autophosphorylation sites involved in MHCK A activation and then determining the effect of actin on versions of MHCK A in which these sites have either been replaced with non-phosphorylatable counterparts or eliminated completely. Such studies may also shed light on the mechanisms by which other factors, such as myosin II and acidic phospholipids, are able to activate MHCK A (26, 27).

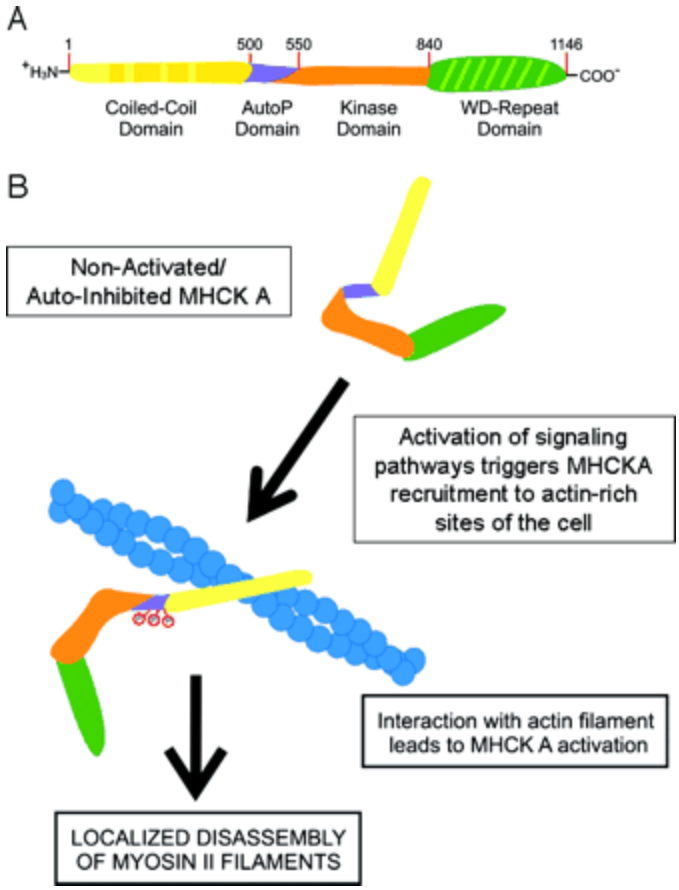

Fig. 6.
Proposed model for actin-mediated stimulation of MHCK A catalytic activity. *A*, structural domains of MHCK A. The *yellow shaded regions* of the amino-terminal coiled-coil domain represent areas exhibiting a high probability to fold into α-helical coiled-coil structures based upon analysis of the sequence with the COILS algorithm (61). The *purple-colored* region represents the putative autophosphorylation (*AutoP*)/autoinhibitory domain of the kinase (45). The central kinase catalytic domain (in *orange*) is the founding member of a novel family of serine/threonine kinases (28, 30). The carboxyl-terminal domain (in *green*) contains a 7-fold WD repeat motif that is characteristic of the β-subunit of heterotrimeric G-proteins (62); this domain facilitates MHCK A-mediated phosphorylation of myosin heavy chain by physically targeting the kinase directly to myosin II filaments (33). *B*, MHCK A in the non-activated state exhibits minimum levels of activity due to decreased substrate accessibility to the catalytic core imposed by the putative auto-inhibitory domain of the enzyme. Upon activation of signaling pathways associated with processes such as chemoattractant stimulation of cells, cytokinesis, or pseudopod formation (21, 22, 35), MHCK A is recruited to actin-rich sites where the coiled-coil domain of the kinase can interact directly with actin filaments. This interaction results in stimulation of MHCK A activity and localized disassembly of myosin II filaments and/or prevention of myosin II assembly at such sites. Physiologically, these events have the potential for contributing not only to the polarized distribution of myosin II filaments during various cellular events (9) but also to the localized relaxation of the actomyosin cytoskeleton during the initial stages of pseudopodia formation (60).

Within the cell, regulation of protein kinase function can be achieved in a number of ways. For example, selective targeting of protein kinase C isoforms to specific subcellular destinations, such as the cytoskeleton, has been shown to be an important mechanism for coordinating the activities of the protein kinase C family of kinases whose members exhibit subtle but relatively broad substrate specificity (46, 47). A well documented example of such regulation has come from detailed cellular and biochemical studies of a protein kinase Cϵ isoform that is recruited to the cytoskeleton via the activity of a well defined actin binding domain (48, 49). Studies in PC12 cells indicate that the increased localization and associated activation of protein kinase Cϵ at actin-rich cortical sites during neuronal cell differentiation may play a critical role in mediating neurite outgrowth in these cells (50). Most interestingly, a recently described bacterial protein kinase (YpkA) from *Yersinia* represents another example of an actin-activated serine/threonine kinase. In the case of YpkA, the enzyme is activated upon interaction with actin filaments in the cytosol of a eukaryotic host cell invaded by the bacterium; the increased YpkA activity leads to disruption of the actin cytoskeleton and the breakdown of cytoskeleton-dependent processes in the host cell (51). Structure-function studies of YpkA revealed a novel 20-amino acid actin-binding motif that is required for YpkA activation by actin filaments (51).

In *Dictyostelium*, there are at least three related kinases (MHCK A, B, and C) capable of regulating myosin II filament assembly in the cell (22, 52). Functional redundancy among these enzymes may be avoided, in part, by targeting the individual kinases to distinct subcellular locations (21, 22). In the case of MHCK A, regulated translocation to actin-rich regions of the cell cortex is mediated by a domain exhibiting a high probability to form α-helical coiled-coil structures (42). In most proteins, the coiled-coil motif functions mainly as an oligomerization domain in which two or more α-helical structural motifs associate via complementary interaction between well defined regions of hydrophobicity (53). In contrast, our studies of MHCK A have demonstrated that the coiled-coil domain is a multifunctional region of the kinase that mediates not only oligomerization but also is responsible for the subcellular localization and actin-binding activities of MHCK A (Fig. 5) (29, 42). It is possible that the mechanism by which the coiled-coil domain of MHCK A mediates actin-dependent stimulation of MHCK A may involve a mechanism similar to that described for the c-Fes non-receptor tyrosine kinase. Studies of c-Fes have revealed that the amino-terminal coiled-coil domain of this kinase regulates the cellular activity of the kinase by controlling the oligomerization state of the kinase (54). Experiments are in progress to identify the minimal region(s) of the coiled-coil domain of MHCK A involved in mediating the oligomerization, translocation, and actin-binding activities of the kinase.

The actin-binding results presented here are noteworthy because they represent the identification of a potentially unique actin-binding domain that is rich in α-helical structural motifs (Fig. 5). Examination of the predicted secondary structure of the MHCK A molecule indicates that the coiled-coil domain of MHCK A (amino acids 1–498) contains a region of about 350 amino acids that possesses a very high probability of forming coiled-coil structures; stretches of amino acids with little or no predicted coil structure are located upstream and interspersed within this coil-rich region (28, 55). Preliminary structure-function studies indicate that it is the coiled-coil regions of this domain that are involved in actin-binding because removal of the amino-terminal non-coiled part of the coiled-coil domain does not alter the interaction with F-actin.[2] Thus, it seems possible that the coiled-coil domain of MHCK A may bind to F-actin via a mechanism similar to that of tropomyosin, a well studied actin-binding protein possessing almost complete coiled-coil structure (56).

Tropomyosin forms two-stranded, parallel coiled-coils; each tropomyosin dimer binds along seven actin monomers in the long pitch groove of the actin filament helix (57). Most interestingly, the stoichiometry of the tropomyosin-actin interaction (0.14 mol of tropomyosin/mol of actin) is similar to that estimated for actin binding by the coiled-coil domain of MHCK A (0.16 mol of GST-Coil/mol of actin; Fig. 5). Moreover, previous analysis of the MHCK A molecule by rotary-shadowing imaging (29), along with estimations based on a theoretical rise of about 0.15 nm per amino acid in a right-handed α-helix (58), indicates that the length of the coil-rich region of MHCK A (about 50 nm) is similar in length to the tropomyosin dimer (41 nm) (56). Despite these similarities, it is still unclear if MHCK A indeed interacts with F-actin via a mechanism similar to that described for tropomyosin because there are significant differences between these two structures. For example, the coiled-coil domain of MHCK A, in contrast to tropomyosin, is predicted to form three-stranded coiled-coil structures (55). In addition, the predicted coiled-coil regions of MHCK A are separated by short stretches of amino acids not predicted to have coiled structure; by contrast, the tropomyosin molecule possesses essentially uninterrupted coiled-coil structure (59). Studies are in progress to better define the characteristics of the interaction between the coiled-coil domain of MHCK A and F-actin.

In summary, the studies described here point to a new concept for cytoskeletally based spatial control of protein kinase activity, in which the protein kinase must localize to F-actin structures before it can become activated. The presence of a similar mechanism in *Yersinia* YpkA (51) suggests that this could be a widespread mode of regulation that might well have been overlooked previously for other cytoskeletally associated protein kinases. In the context of *Dictyostelium* cell movements, our data establishing that MHCK A is regulated by interaction with F-actin provides further insight on how the cell is able to integrate many signals to give specific changes in cell morphology required for cell survival and multiplication. Based on our studies, we propose that the localization of MHCK A to actin-rich sites is likely to be coupled with actin-induced stimulation of kinase activity and thus provides a potent mechanism for the rapid disassembly (or prevention of assembly) of myosin

II bipolar filaments at specific regions of the cell cortex. In turn, localized recruitment and activation of MHCK A may provide a mechanism for targeting relaxation of the actomyosin cortex and thus possibly facilitate the formation of cellular protrusions involved in processes such as chemotaxis, fluid/particle phase uptake, and the final stages of cytokinesis (9, 60). Studies in progress are focused on exploring the structural aspects of MHCK A regulation in the cell with the goal of understanding how the cell is able to coordinate the activities of MHC kinases and phosphatases involved in regulating myosin II filament assembly in the dynamic context of a non-muscle cell.

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Footnotes

- 1 The abbreviations used are: MHC, myosin II heavy chain; MHCK, myosin heavy chain kinase; GST, glutathione *S*-transferase; DTT, dithiothreitol; IPTG, isopropyl β-D-thiogalactopyranoside; TES, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulfonic acid; Lat A, latrunculin A; PBS, phosphate-buffered saline.

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