Journal of Cellular Biochemistry 88:521–532 (2003)

Myosin Heavy Chain Kinase B Participates in the Regulation of Myosin Assembly Into the Cytoskeleton

Maribel Rico and Thomas T. Egelhoff*

Department of Physiology and Biophysics, Case Western Reserve University, Cleveland, Ohio 44106-4970

Abstract Myosin II plays critical roles in events such as cytokinesis, chemotactic migration, and morphological changes during multicellular development. The amoeba *Dictyostelium discoideum* provides a simple system for the study of this contractile protein. In this system, myosin II filament assembly is regulated by myosin heavy chain (MHC) phosphorylation in the tail region of the molecule. Earlier studies identified an alpha-kinase, MHC kinase A (MHCK A), which phosphorylates three mapped threonine residues in the myosin tail, driving myosin disassembly. Using molecular and genomic approaches, we have identified a series of related kinases in *Dictyostelium*. The enzyme MHCK B shares with MHCK A a domain organization that includes a highly novel catalytic domain coupled to a carboxyl-terminal WD repeat domain. We have engineered, expressed, and purified a FLAG-tagged version of the novel kinase. In the present study, we report detailed biochemical and cellular studies documenting that MHCK B plays a physiological role in the control of *Dictyostelium* myosin II assembly and disassembly during the vegetative life of *Dictyostelium* amoebae. The presented data supports a model of multiple related MHCKs in this system, with different regulatory mechanisms and pathways controlling each enzyme. J. Cell. Biochem. 88: 521–532, 2003.

Key words: myosin; chemotaxis; cytokinesis

Eukaryotic protein kinases represent one of the largest families of proteins known. Two major families are traditionally cited: the Ser/Thr and Tyr kinases and the histidine kinases. These two families are unrelated, the histidine kinase superfamily comprise the two component systems of response elements and signal transducers, and are frequently though not exclusively found in prokaryotes. The diagnostic feature of the eukaryotic Ser/Thr and Tyr kinases is the occurrence of 11 distinct sequence motifs that are critical for the organization of a tertiary structure that allows catalytic activity [Hanks and Quinn, 1991].

Recently, a new family of protein kinases has been characterized that has no detectable sequence similarity to conventional protein kinases and that presents a distinct catalytic domain with conserved motifs that differ from those of the Ser/Thr and Tyr kinases. Two members of this novel family have been extensively studied: the *Dictyostelium discoideum* myosin heavy chain kinase A (MHCK A) [Côté et al., 1997; Egelhoff and Côté, 1999; Luo et al., 2001; Steimle et al., 2001b] and the eukaryotic elongation factor 2 (eEF-2) kinase [Ryazanov et al., 1997, 1999; Pavur et al., 2000]. MHCK A phosphorylates three threonines at positions 1823, 1833, and 2029 in the carboxy-terminal domain of the *D. discoideum* myosin II, promoting the disassembly of myosin II filaments. The eEF-2 kinases phosphorylate threonines 56 and 58 of eEF-2 [Price et al., 1991; Redpath et al., 1993], which inhibits the elongation phase of protein synthesis. The target sites of MHCK A and eEF-2 kinase are both predicted to be held in secondary alpha-helical structures. For this reason, Ryazanov et al. [1999] have suggested that the members of this novel family, unlike conventional kinases [Pinna and Ruzzene, 1996], may display a tendency to phosphorylate their substrates within alpha-helices. Hence, the name of alpha-kinase family has been proposed. Other members of the alpha-kinase family, the channel-kinases 1 and 2 (ChaK1 and ChaK2; [Runnels et al., 2001]), have

Grant sponsor: NIH (to TTE); Grant number: GM50009.

*Correspondence to: Thomas T. Egelhoff, Department of Physiology and Biophysics, Case Western Reserve University, Cleveland, OH 44106-4970.
E-mail: tte@po.cwru.edu

Received 8 August 2002; Accepted 29 August 2002
DOI 10.1002/jcb.10361

attracted great interest lately due to the unprecedented novelty of being fused to an ion channel. Surprisingly, the tertiary structure of one crystallized alpha-kinases catalytic domain revealed partially similarity in one subdomain to a segment of the Ser/Thr and Tyr kinases [Yamaguchi et al., 2001].

Genes encoding alpha-kinase family members are recognizable in genome projects of a variety of organisms including human, mouse, zebrafish, nematodes, and *Dictyostelium* but are notably absent in genome projects of *Saccharomyces cerevisiae*, *Arabidopsis thaliana*, *Schizosaccharomyces pombe*, or *Drosophila melanogaster* [Ryazanov, 2002]. Of particular interest as potential source of pathological disorders, are three new alpha-kinases found in the human genome: the heart alpha-kinase, the muscle alpha-kinase, and the lymphocyte alpha-kinase [Ryazanov, 2002]. Thanks to the *Dictyostelium* genome project efforts, a total of five members are now clearly present in the amoeba *D. discoideum* (our unpublished data). Of these five members found in *Dictyostelium* genome, three of them, MHCK A, MHCK B, and MHCK C showed dramatic preference for Thr over Ser as their phosphoacceptor residue. It has been proposed that the entire alpha-kinase family may be largely threonine-specific [Luo et al., 2001].

Dictyostelium provides thus a simple scenario where, unlike in mammalian non-muscle cells, the control over myosin II filament assembly and disassembly relies primarily on MHC phosphorylation [Egelhoff et al., 1993; de la Roche and Côté, 2001]. In this system, myosin II filaments, driven by their strong affinity towards polymerized actin, localize to the cytoskeleton where they serve as substrate for MHC kinases (MHCKs). MHCKs activity would render phosphorylated myosin monomers that would subsequently localize to the cytosol. Removal of these phosphates by MHC-specific phosphatase activity promotes myosin II filament assembly and consequent filament localization to the cell cortex [Murphy and Egelhoff, 1999].

Earlier work in our laboratory using molecular cloning techniques, identified a gene containing a catalytic region with high homology to the catalytic domain of MHCK A. Heterologous expression of this novel catalytic region and in vitro biochemical studies [Clancy et al., 1997] clearly demonstrated its potential role as a MHCK. Furthermore, analysis of the domain structure revealed strong homology to that of MHCK A and hence it was named MHC kinase B (MHCK B). In vitro analysis of bacterially produced subdomains of MHCK B suggested that myosin was its true physiological substrate [Steimle et al., 2001a].

In order to investigate and characterize the biochemical parameters of full-length, native MHCK B, we have engineered, expressed, and purified a FLAG-tagged version of the kinase. In this study, through the analysis of overexpression and knocked out mutants, we provide biochemical and cellular data indicating that MHCK B plays a physiological role in the control of *Dictyostelium* myosin II assembly and disassembly.

EXPERIMENTAL METHODS

Plasmid Construction and Cell Lines

A full-length cDNA clone encoding the entire MHCK B open reading frame was created from previously reported overlapping cDNA clones [Clancy et al., 1997]. PCR was used to engineer an EcoRI restriction site adjacent to codon 2 of the MHCK B coding region (GenBank Accession P90648). This cDNA was then subcloned into pTX-FLAG [Levi et al., 2000], fusing a FLAG epitope at the amino-terminus of MHCK B, creating the plasmid pTX-MKB2. Insert expression in this extrachromosomal vector is driven from the strong constitutive actin 15 promoter.

FLAG-MHCK B overexpressing cell lines (MHCK B^{OE} cells) were obtained by electroporating pTX-MKB2 into either Ax2 cells (expressing wild type myosin) or 3× ALA cells (containing 3× ALA myosin [Egelhoff et al., 1993]) with selection in HL5 medium at 10 μg/ml G418. Once clonal cell lines were obtained, selection level was increased to 25 μg G418/ml to increase FLAG-MHCK B expression level, as described previously for expression of MHCK A [Kolman and Egelhoff, 1997]. The MHCK B gene-targeting construct was generated as follows. The plasmid pFUNK1 contains the entire *mhkB* cDNA cloned as an EcoRI fragment in the vector pGEM7. This plasmid was restricted with BsaB1 and EcoRV, which removes an 871-bp segment from the center of the coding region. A blasticidin resistance cassette from the plasmid pBSR-Nsi (excised as a BamHI–HindII fragment) was swapped into the pFUNK1 plasmid via blunt end ligation,

replacing the central portion of *mhkB* with the Bsr-resistance cassette. The final targeting construct, pUNK-KO, retains 530 bp of targeting homology at the 5′ end and 870 bp of targeting homology at the 3′ end of the construct. The plasmid pBSR-Nsi was generated from the plasmid pBsr-delta-Bam [Adachi et al., 1994] by restriction digestion with NsiI and blunt-ended religation to eliminate a tandem reiteration present in the actin 8-terminator segment of pBsr-delta-Bam.

Gene targeting was performed by linearizing pUNK-KO at flanking restriction sites and electroporating into *Dictyostelium* cell line JH10 [Hadwiger and Firtel, 1992] followed by selection in HL5 medium [Sussman, 1987] supplemented with blasticidin [Adachi et al., 1994]. Analysis of four independent clonal cell lines via Western blotting revealed a loss of *mhkB* expression in all four lines. Genomic PCR analysis performed on these cell lines indicated complete replacement of the native *mhkB* gene by the targeting construct.

SDS–PAGE protein lysates for developmental expression analysis were produced by developing *Dictyostelium* Ax2 cells on buffered filter pads as described previous [Sussman, 1987], with minor modifications. Filter pads were placed on the surface of starvation buffer agar plates (20 mM MES [pH 6.8], 0.2 mM $CaCl_2$, 2 mM $MgCl_2$, 1.5% agar), and cells were spotted on these filters for the standard time points, 0,4,8,12,16, and 24 h. Cells were collected at each time point by washing cells off the filter with 2× SDS–PAGE sample buffer into microfuge tubes. Samples were then heated to 100°C for 60 s before analysis by SDS–PAGE and Western blotting. Growth rates in suspension culture were determined by transferring plate-grown to flasks containing HL5 media at low density (5×10^4 cells/ml). Cell density was determined daily via hemacytometer counts.

FLAG MHCK B protein purification. MHCK B overexpression cell lines were grown in suspension in HL5 media. Typically, 12 L yielded ~50 g of cells. Collection of cells and all subsequent steps were performed at 0–4°C. Cells were washed then lysed in lysis buffer (50 mM Tris pH 8.0; 1 mM EDTA; 1 mM DTT) containing 1 × protease inhibitor cocktails (PIC I and PIC II) [Steimle et al., 2001a] by sonication in an ice water bath. Sodium chloride was then added to 150 mM, which was necessary to prevent MHCK B from associating with the particulate material in the cell lysate. The resulting lysate was centrifuged at 2,000 rpm for 5 min. To the cleared supernatant ammonium sulfate was added to 30% saturation. This lysate was subjected to centrifugation to remove the 30% ammonium sulfate precipitate, and to the supernatant ammonium sulfate was added to 50% of saturation. MHCK B, which precipitates in this 30–50% ammonium sulfate cut, was collected by centrifugation at 15,000 rpm for 20 min. The resulting pellet was resuspended by douncing in binding buffer (50 mM Tris pH 7.5; 150 mM NaCl; 1 mM EDTA, containing PIC I and PIC II), and incubated in batch with 2 ml anti-FLAG agarose resin (Sigma, St. Louis, MO) for 1 h at 4°C. The beads were washed several times with binding buffer to remove unbound material, then poured into a disposable column (BioRad, Richmond, CA). An additional wash with binding buffer + PICs containing 20 μg/ml FLAG peptide was performed to remove non-specifically bound material. FLAG-MHCK B was released from the beads by treatment with 1–2 ml of elution buffer (50 mM Tris pH 7.5; 150 mM NaCl; 1 mM EDTA;120 μg/ml FLAG peptide) + PICs. A 10–20% reduction in volume was obtained by dehydration with Aquacide (Calbiochem, San Diego, CA). Dialysis against 1 L of storage buffer (20% glycerol; 20 mM Tris pH 7.5 20 mM KCl; 1 mM EDTA; 1 mM DTT) was performed for 3 h at 4°C. Aliquots were frozen on dry ice and stored at −80°C.

Biochemical Methods

Rabbit polyclonal anti-MHCK B antisera was generated by immunizing rabbits with the purified MHCK B catalytic domain that was expressed in bacteria and purified using a 6 × histidine tag. Western blot experiments with this antisera were performed using CDP-star reagent (Tropix, Bedford, MA) chemiluminescence detection system. Purified FLAG-MHCK B was subjected to SDS–PAGE along with known concentrations of BSA to create a standard curve. After Coomassie blue staining, the protein bands were quantified by scanning densitometry and the unknown concentrations determined by extrapolation from the BSA standards.

Myosin phosphorylation, MH1 peptide phosphorylation, and autophosphorylation assays were performed at 22°C in a water bath in kinase buffer (10 mM TES pH 7.0, 1 mM DTT,

2 mM $MgCl_2$, and 0.5 mM [γ-^{32}P]-ATP with an specific activity of 400–800 Ci/mole). The MH1 peptide (RKKFGESEKTKTKEFL) described previously [Medley et al., 1991], corresponds to the mapped MHCK A target site in the myosin II heavy chain tail at residue 2029 (underlined threonine). The MH1 peptide was used as substrate for the study of kinetic parameters at the concentrations indicated in the corresponding figures. Reactions using MH1 peptide were stopped by addition of EDTA to 20 mM final concentration. The amount of γ-^{32}P incorporated into the peptide molecules was quantified by spotting reactions onto P-81 filters (Whatman, Maidstone, England) as described [Medley et al., 1991]. Purified *Dictyostelium* myosin II (0.5 μM) was used to determine the stoichiometry of MHC phosphorylation. Throughout this work, all myosin II phosphorylations were performed with native myosin II, but concentrations are expressed in terms of moles MHC present in the reaction. Myosin II concentrations were quantified from SDS–PAGE as described above for MHCK B quantification. Reactions using myosin II as substrate and MHCK B autophosphorylation reactions were stopped by addition of 5× SDS–PAGE sample buffer and heating at 90°C for 60 s. Quantification was performed by SDS–PAGE, Coomassie staining of the gels, and scintillation counting of the bands of interest.

Myosin assembly following MHCK B phosphorylation was assessed with an initial phosphorylation step (60 nM MHCK B, 0.8 μM myosin, 0.5 mM ATP, 2 mM $MgCl_2$, 10 mM TES pH 7.0, 1 mM DTT; 15 min reaction at 22°C; stopped by addition of 20 mM EDTA final followed by adjustment to 50 mM NaCl to provide optimal conditions for myosin filament formation. Samples were then centrifuged at 55,000 rpm for 10 min. The pellets were resuspended in 2× SDS–PAGE sample buffer and the supernatants subjected to TCA precipitation and resuspended in 2× SDS–PAGE sample buffer. Supernatant and pellet fractions were subjected to SDS–PAGE and Coomassie staining. Relative amounts of myosin were quantified by scanning densitometry.

To evaluate myosin assembly into Triton X-100-insoluble cytoskeletal fractions, 1.5×10^6 cells were harvested and washed in 10 mM TES pH 7, 50 mM KCl, then resuspended in Buffer A (100 mM MES pH 6.8, 2.5 mM EGTA, 5 mM $MgCl_2$, 0.5 mM ATP). An equal volume of Buffer B (1% Triton X-100, 100 mM MES pH 6.8, 2.5 mM EGTA, 5 mM $MgCl_2$, 0.5 mM ATP, 1× PIC I and PIC) was then added and tubes were vortexed at medium speed for 30 s, then centrifuged at maximum speed in a microfuge for 1 min. The cytoskeletal pellets were then resuspended in 2× SDS–PAGE sample buffer. The supernatants were subjected to acetone precipitation, and resuspended in 2× SDS–PAGE sample buffer. Supernatants and pellet samples were subjected to SDS–PAGE and relative amounts of myosin quantified by scanning densitometry.

RESULTS

MHCK B-Null and Overexpression Mutants

The domain organization of MHCK A and MHCK B have in common the presence of an alpha-kinase catalytic domain, and the presence of a carboxyl-terminal WD-repeat domain (Fig. 1A). MHCK A also contains an amino-terminal domain of ~70 kDa that was shown

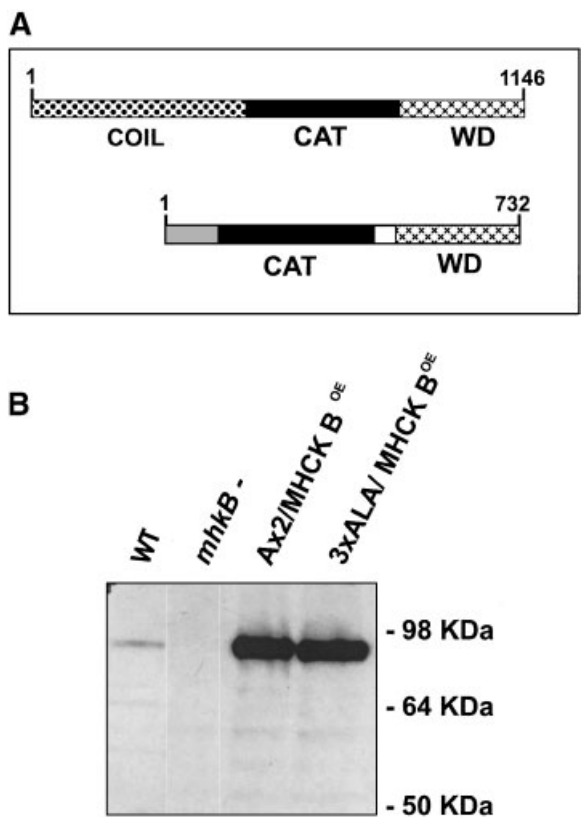

Fig. 1. **A**: Domain structure of *Dictyostelium discoideum* myosin heavy chain kinase A (MHCK A, top) and myosin heavy chain kinase B (MHCK B, lower). **B**: Western blot analysis of MHCK B expression in the cell lines used for this study.

previously to drive homo-oligomerization of MHCK A [Kolman and Egelhoff, 1997]. MHCK B does not contain any strongly predicted coiled-coil segments.

To allow further cellular and biochemical analysis of the roles of MHCK B, we have generated MHCK B overexpression cell lines (MHCK B^{OE}) and gene disruption cell lines (*mhkB*$^-$) in *Dictyostelium*. The MHCK B null cell lines were confirmed by genomic PCR (not shown) and display no signal on a Western blot using a polyclonal anti-MHCK B antisera raised against the catalytic domain of the enzyme (Fig. 1B). Western blot coupled to densitometric analysis of the MHCK B^{OE} cell lines indicated an overexpression of at least 75-fold for MHCK B (Fig. 1B).

To gain insights into what setting or settings MHCK B might have cellular roles, we evaluated the developmental expression pattern of the enzyme. Western blot analysis revealed expression during vegetative growth and throughout the time course of multicellular development (data not shown). This expression pattern suggests that MHCK B may participate in myosin II assembly control during both growth phase and developmental motility events.

MHCK B Purification and Biochemical Analysis

Myosin II null cell lines expressing the phosphorylation resistant 3× ALA myosin II (described later), and overexpressing the FLAG-tagged *mhkB* gene (3× ALA/MHCK B^{OE} cells) were grown in suspension culture to provide sufficient yield for biochemical purification. FLAG-MHCK B was purified by sonication of washed cells followed by ammonium sulfate fractionation that yielded a strong enrichment of FLAG-MHCK B in a 30–50% ammonium sulfate fraction. The resulting ammonium sulfate precipitate was resuspended and subjected to affinity purification by using anti-FLAG IgG-agarose resin. This procedure yielded a highly purified FLAG-MHCK B as determined by SDS–PAGE and Coomassie staining (Fig. 2A) and by Western blot analysis (Fig. 2B). The expressed protein migrates at ~98 kDa on SDS–PAGE, reasonably similar to the predicted mass of 85 kDa for the epitope tagged form of MHCK B. FLAG-MHCK B phosphorylated native *Dictyostelium* myosin II efficiently (Fig. 2C and as described further below), up to a stoichiometry similar to that reported previously for bacterially expressed MHCK A and B catalytic domains under similar reaction conditions [Steimle et al., 2001a].

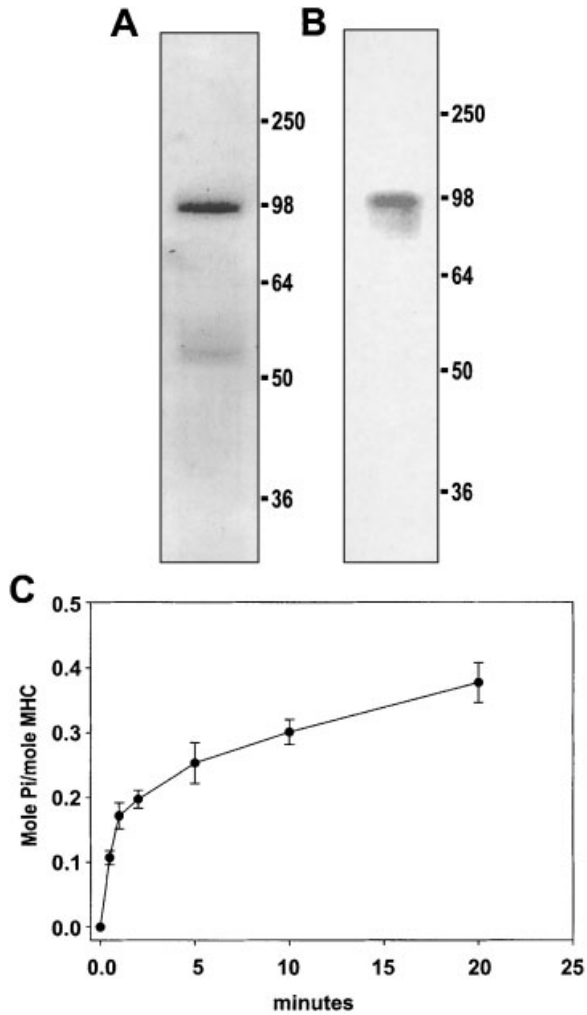

Fig. 2. FLAG-MHCK B purification. **A**: Coomassie stained SDS–PAGE of the affinity purified FLAG-MHCK B. **B**: Western blot of purified MHCK B. **C**: Myosin phosphorylation by affinity purified FLAG-MHCK B, reaction performed with 20 nM MHCK B and 0.5 μM myosin. Bar, SE of mean, n = 3.

For many protein kinases, autophosphorylation leads to increased activity. For MHCK A autophosphorylation raises the V_{max} by greater than 50-fold [Medley et al., 1990], and similar in vitro activation has been observed for MHCK C [Liang et al., 2002]. We studied the effect of autophosphorylation on MHCK B activity by performing in vitro kinase assays. Our result shows that FLAG-MHCK B autophosphorylates up to 3.5 moles Pi/mole kinase (Fig. 3A). In contrast to MHCK A, MHCK B autophosphorylation did not significantly enhance its activity towards the MH1 peptide (Fig. 3A, inset).

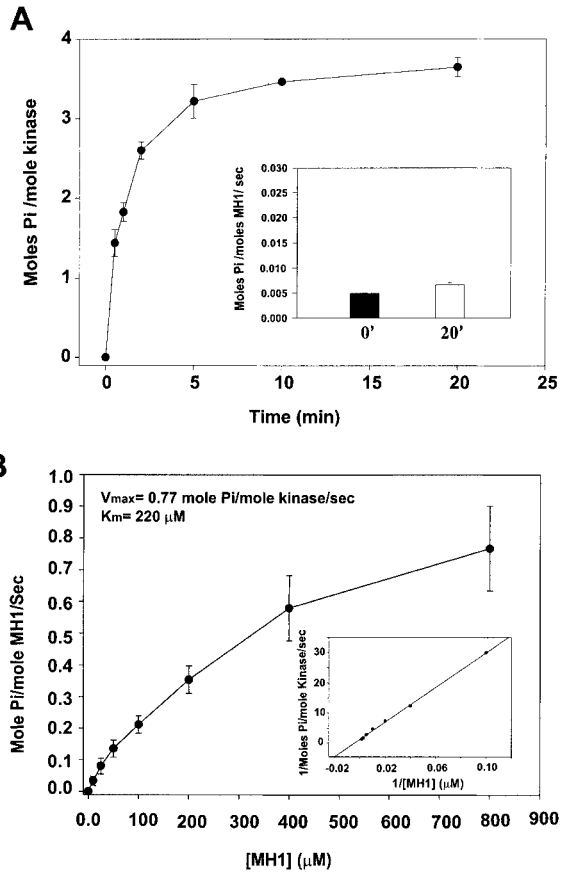

Fig. 3. Biochemical parameters of FLAG-MHCK B. **A**: Stoichiometry of MHCK B autophosphorylation assayed at 60 nM kinase. Inset: autophosphorylation does not affect FLAG-MHCK B activity towards the MH1 peptide. Purified FLAG-MHCK B (20 nM) was used to phosphorylate MH1 peptide (50 μM) in a 1 min reaction. MHCK B was either added directly to the reaction or allowed to autophosphorylate for 20 min before initiating MH1 phosphorylation test. **B**: Kinetics analysis of MHCK B activity towards MH1 peptide. Insert: Lineweaver–Burke representation.

Under standard in vitro kinase assay conditions, (2 mM Mg^{2+}, 0.5 mM ATP) *Dictyostelium* myosin II is mostly filamentous. For this reason myosin II as a substrate is not compatible with the study of Michaelis–Menten kinetic parameters. These parameters were, therefore, studied by using a synthetic peptide substrate (MH1) that had been used earlier for the study of the kinetic analysis of MHCK A [Medley et al., 1991]. This 16-residue peptide is equivalent to a section of the MHC centered on threonine 2029, a major target for MHCK A activity in the native myosin. The MH1 peptide is predicted to be a suitable substrate for MHCK B because of its established preference for threonine as phosphoacceptor residue [Luo et al., 2001]. FLAG-MHCK B displayed a V_{max} of 0.77 mole Pi/mole kinase/s and a K_M of 220 μM with the MH1 substrate (Fig. 3B). These values are similar to those obtained in earlier studies with native MHCK A [Medley et al., 1990] and with recombinant MHCK A and MHCK B catalytic domains [Steimle et al., 2001a]. These biochemical results support the idea that myosin II is the physiological substrate for MHCK B activity and that MHCK B may contribute to the control of myosin II assembly in vivo.

MHCK B Activity Disrupts In Vitro Myosin Filament Formation

In vivo phosphorylation of the MHC occurs primarily on threonine and serine residues [Berlot et al., 1987; Egelhoff et al., 1993]. MHCK A specifically phosphorylates three threonines in the tail region of MHC to drive filament disassembly. We performed in vitro myosin phosphorylation followed by a sedimentation assay in order to determine whether or not myosin phosphorylation by MHCK B blocks myosin filament formation. The sedimentation assay was carried out at 50 mM NaCl to provide optimal ionic strength for filament formation. In these conditions non-phosphorylated myosin monomers will be predominantly filamentous and consequently, sediment. In contrast, phosphorylated myosin remains non-filamentous and localizes to the supernatant. A parallel kinase assay containing [γ-^{32}P]-ATP was performed in order to quantify the stoichiometry of phosphorylation. Under these conditions MHCK B phosphorylated myosin to a stoichiometry of 1.8 moles Pi/mole MHC, and reduced the level of sedimenting (filamentous) myosin from 80 to 24% (Fig. 4) providing additional evidence for the role of MHCK B in phosphorylation-based myosin II disassembly. The notably higher stoichiometry of myosin phosphorylation in this assay relative to Figure 2 is attributable to the increased concentration of both substrate and kinase under these assay conditions.

Overexpression of MHCK B Results in Cytokinesis Defects

In *Dictyostelium* cells, failure in myosin II function, due either to gene disruption or to mutations in the myosin gene, leads to a failure in cytokinesis in suspension culture, resulting in a characteristic multinucleated cell phenotype that eventually leads to cell lysis [De

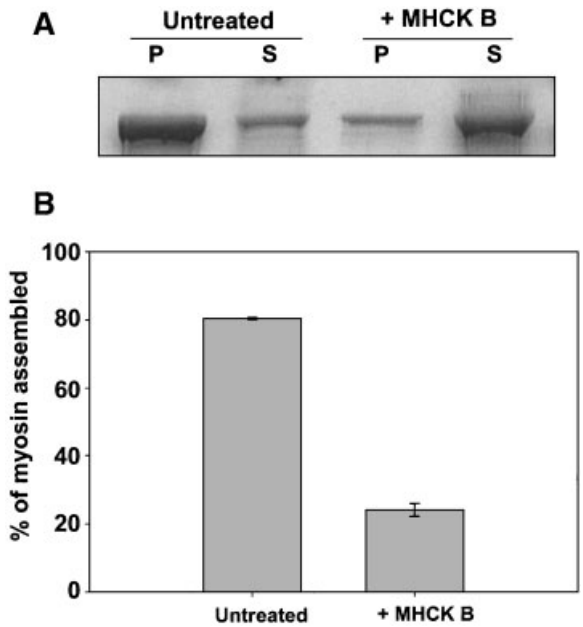

Fig. 4. MHCK B phosphorylation of myosin drives filament disassembly in vitro. *Dictyostelium* myosin (800 nM) was either mock phosphorylated (untreated) or phosphorylated for 15 min with 60 nM MHCK B. After the phosphorylation step samples were adjusted to 50 mM NaCl to optimize filament assembly and subjected to centrifugation to pellet assembled myosin. **A**: Coomassie staining showing MHC band in pellet (P) and supernatant (S) fractions. **B**: Quantification of assembly levels determined by densitometry of Coomassie stained gels. Bars = SE, n = 3.

Lozanne and Spudich, 1987]. We utilized this distinctive phenotype as a reporter for whether overexpression of MHCK B could drive myosin II filament disassembly in vivo.

The multicopy plasmid pTX-MKB2 has a strong constitutive promoter driving expression of full-length MHCK B with a FLAG epitope tag at the amino-terminus. This plasmid was transfected into the *Dictyostelium* strain Ax2, and into previously described 3× ALA myosin cell line, which constitutively overassemble myosin II [Egelhoff et al., 1993]. Clonal cell lines were selected and cultured (referred to as Ax2/MHCK B^{OE} and 3× ALA/MHCK B^{OE} cells, respectively).

Ax2 cells transfected with empty FLAG vector (Fig. 5A, open squares) grew in suspension culture to densities similar of the parental Ax2 cells; these cells also remained generally mono or binucleated (Fig. 5B,C) as expected for cells capable of proper myosin assembly and cytokinesis in suspension. In contrast, Ax2/MHCK B^{OE} cells displayed reduced ability to increase their cell density in suspension culture, with cell counts typically around 10^6 cells/ml after a week (Fig. 5A, open circles), a tenfold lower cell density than that observed for control cells. Nuclear DAPI staining of Ax2/MHCK B^{OE} cells collected from suspension culture revealed a marked increase in the average number of nuclei per cell (Fig. 5C). When grown as surface-attached petri dish cultures, Ax2/MHCK B^{OE} cells did not display this multinucleated phenotype (not shown) indicating that this defect is specific to suspension culture growth. These results are consistent with the hypothesis that overexpression of MHCK B results in disassembly of myosin II filaments in vivo, inducing a myosin null-like defect in cytokinesis in suspension.

The effects of MHCK B overexpression in Ax2 cells contrast to the effects of overexpression observed in the 3× ALA myosin cell line. The 3× ALA myosin cell line expresses an altered MHC gene in which the mapped target sites for MHCK A have been converted from threonine to alanine residues. In earlier work it was shown that this 3× ALA myosin grossly overassembles into the cytoskeleton due to its resistance to phosphorylation-mediated myosin filament disassembly [Egelhoff et al., 1993]. In the current analysis, we found that the 3× ALA/MHCK B^{OE} cells grown in suspension culture displayed a partial tendency towards multinucleation (Fig. 5B,C), but were capable of growth in suspension culture to densities similar of the control cells (Fig. 5A, filled circles). These results strongly suggest that MHCK B overexpression impairs growth in suspension due to threonine phosphorylation-mediated myosin filament disassembly. Furthermore, they highlight the relevance of these three key threonines at the MHC-carboxy terminus for the control of biological processes depending upon myosin II function in the vegetative amoebas of *D. discoideum*.

MHCK B Overexpression Reduces Cytoskeletal Myosin II Levels In Vivo

The amount of myosin II filaments in the cytoskeleton of living amebas can be quantified by dissolution of the cell membrane with the detergent Triton X-100, which extracts myosin monomers, but which leaves assembled cortical myosin filaments in the particulate fraction [Spudich, 1987]. In earlier work, overexpression of MHCK A in *Dictyostelium* cell lines bearing wild type myosin significantly reduced the

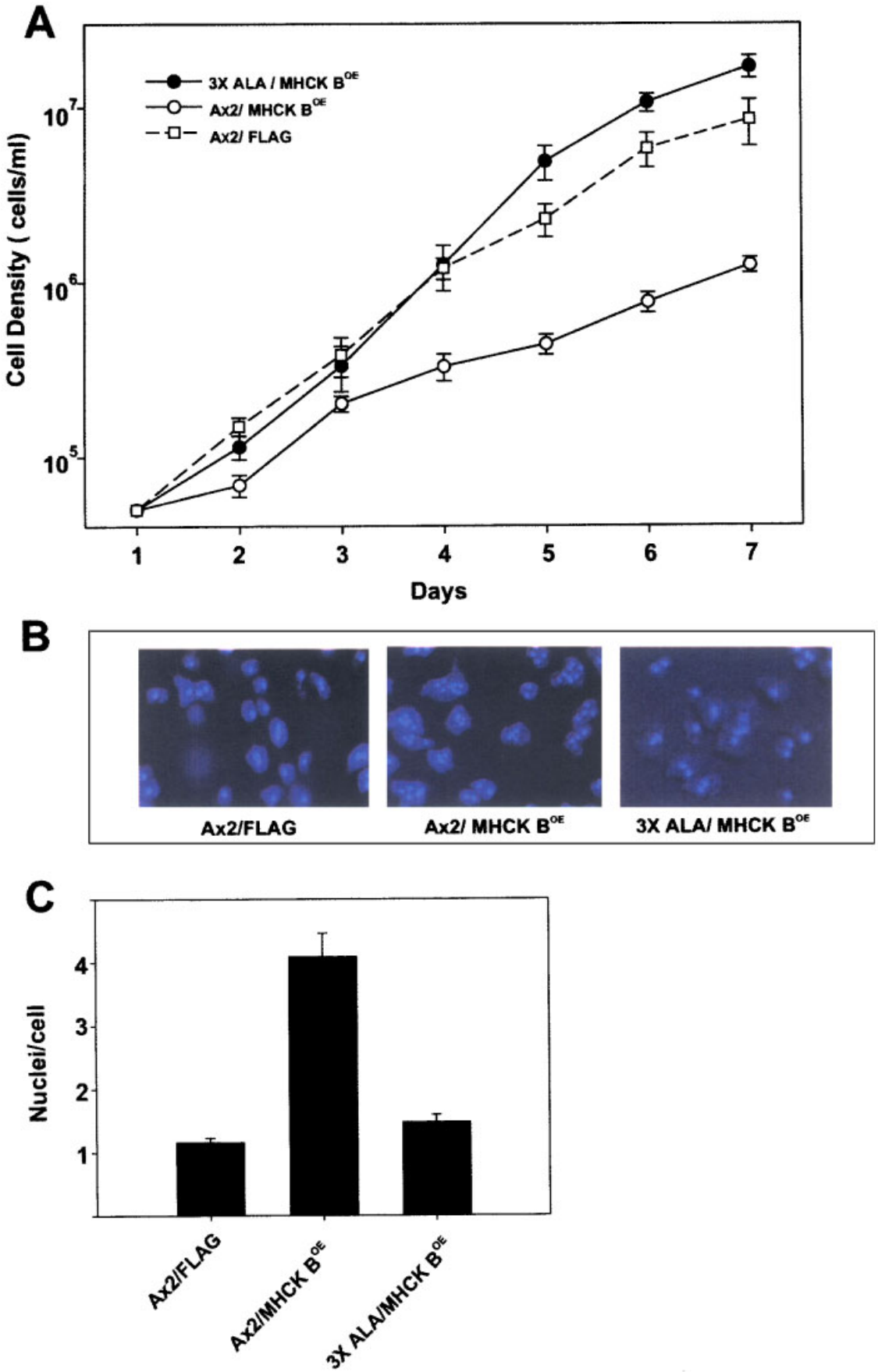
A
3X ALA / MHCK B^OE
Ax2/ MHCK B^OE
Ax2/ FLAG
Cell Density (cells/ml)
10^7
10^6
10^5
1
2
3
4
5
6
7
Days
B
Ax2/FLAG
Ax2/ MHCK B^OE
3X ALA/ MHCK B^OE
C
Nuclei/cell
4
3
2
1
Ax2/FLAG
Ax2/MHCK B^OE
3X ALA/MHCK B^OE

Fig. 5.

amount of myosin present in the Triton-insoluble particulate fraction [Kolman et al., 1996], while disruption of the MHCK A gene resulted in a partial overassembly of myosin into this fraction.

To perform similar analysis with MHCK B, we assessed the behavior of Ax2/FLAG and Ax2/MHCK B^{OE} cells using this assay. In these tests, the isogenic control cell line, Ax2/FLAG showed 12% of the myosin II sedimented with the cytoskeletal ghosts, while in Ax2/MHCK B^{OE} cells only 8% of the myosin II sedimented with the cytoskeletal ghosts (Fig. 6; $P < 0.02$). These results, although modest, indicate that an activity promoting myosin disassembly is increased in MHCK B overexpressing cells. The lack of correlation between the level of overexpression and the level of myosin disassembly might be pointing to an additional mechanism controlling MHCK B activity (see Discussion).

Analysis of Myosin II Assembly Levels in MHCK B Null Cell Lines

To further investigate the role of MHCK B in the control of myosin assembly in the cytoskeleton we engineered cell lines in which the *mhkB* gene is eliminated via gene targeting. A gene targeting plasmid was constructed in which most of the *mhkB* coding region is replaced with a blasticidin antibiotic resistance cassette. This construct was transfected into *Dictyostelium* strain JH10, and resulting blasticidin resistant colonies were screened initially by Western blot. Loss of *mhkB* expression was typically observed in 50–70% of the blasticidin resistant clonal cell lines obtained from such transfections (Fig. 1B). Follow-up genomic PCR analysis confirmed that the loss of *mhkB* expression in these cell lines was the result of the disruption of the *mhkB* gene by the targeting plasmid.

In *mhkB* null cells, Triton-insoluble cytoskeletal analysis revealed a partial increase in myosin assembly levels relative to isogenic JH10 cells transfected with an empty Bsr-R plasmid (JH10*bsr* cells; Fig. 7), from ~11% of myosin associated with the cytoskeletal fraction

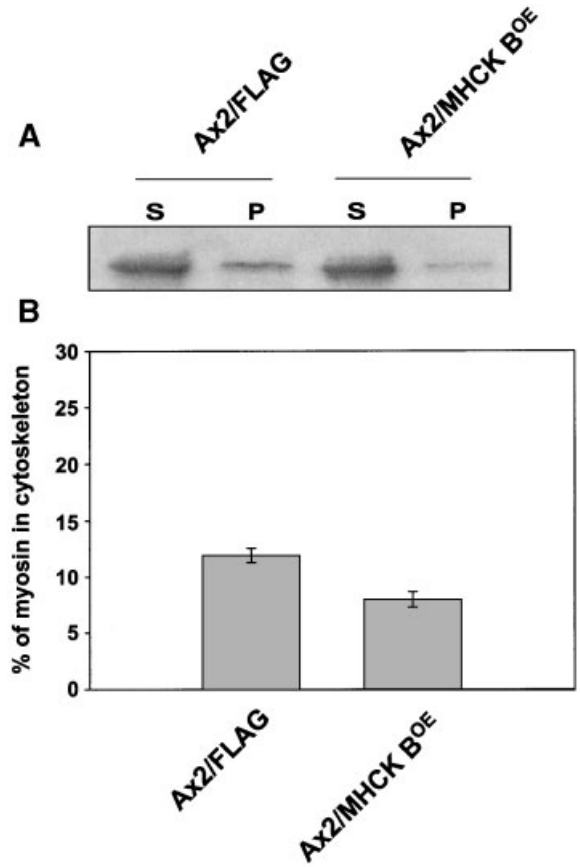

Fig. 6. Ax2/MHCK B^{OE} cells have reduced levels of myosin II assembled into in Triton X-100-insoluble cytoskeletal fractions of cell lysates. **A**: Western blot showing the amount of myosin for Ax2/FLAG and Ax2/MHCK B^{OE} cell lines in the soluble supernatant fraction (S) and in the Triton-insoluble cytoskeletal fraction (P). **B**: Quantification of myosin II present in Triton-insoluble fractions in each cell line, determined from densitometry of Western blots. Bar, SE of the mean, n = 4. $P < 0.02$.

in JH10*bsr* cells versus 22% of myosin associated with the cytoskeleton in *mhkB* null cells ($P < 0.01$). This strong effect on myosin assembly indicates a physiological role for MHCK B activity on the control of *Dictyostelium* myosin II function.

The level of overassembly in the *mhkB* null cell lines is very similar to that observed in earlier studies where MHCK A was disrupted [Kolman et al., 1996]. This similarity, and the

Fig. 5. Analysis of growth in suspension A. Cells that overexpress MHCK B in the presence of wild type myosin (Ax2/MHCK B^{OE}) show reduced growth in suspension compared to cells that carry the empty vector (Ax2/FLAG). Cells that overexpress MHCK B in the presence of phosphorylation-resistant 3× ALA myosin (3× ALA/MHCK B^{OE}) display growth similar to parental cell line. **B**: DAPI image showing multinucleation in Ax2/MHCK B^{OE}cells relative to control Ax2/FLAG cells and 3 × ALA/MHCK B^{OE} cells. **C**: Quantification of number of nuclei per cell for the three different cell lines (n = 40 cells). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

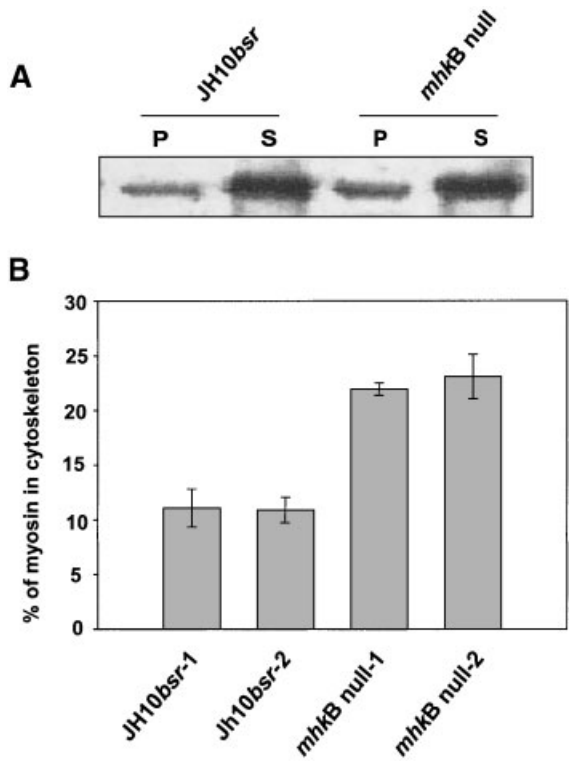

Fig. 7. *mhkB* gene disruption results in increased myosin assembly into the Triton X-100 insoluble fraction of lysed cells. **A**: Anti-myosin II Western blot showing the amount of myosin in pellet (P) and supernatant (S) fractions for isogenic control JH10*bsr* cells and *mhkB* null cells. **B**: Quantification of myosin II present in Triton-insoluble fractions in two independent control cell lines (JH10*bsr*) and two independent *mhkB* null cell lines, determined via densitometry from Western blots. Bar, SE of the mean, with n values of 4–6 in each sample, and $P < 0.01$ for the difference between JH10*bsr* and *mhkB* nulls.

similar lack of severe phenotypic defects in *mhkB* null versus *mhkA* null cell lines [Kolman et al., 1996], suggests that these two enzymes may function in a partially redundant or in an additive manner to control filament assembly levels in vivo.

DISCUSSION

Crystallographic studies on the catalytic domain of the ChaK1 revealed the model structure of the alpha-kinase family catalytic domain [Yamaguchi et al., 2001]. The alpha-kinases are similar to the conventional ser/thr and tyr kinases in the overall organization, with two lobes separated by a cleft. Despite the absence of detectable similarity in the primary sequence, the smaller amino-terminal lobe of the alpha-kinases displays a fold remarkably similar to conventional eukaryotic protein kinases. The ATP binding pocket is located in the inter-lobe cleft in alpha-kinases as well as in conventional kinases. However, the larger carboxyl-terminal lobe, implicated in substrate binding [Pinna and Ruzzene, 1996] differs dramatically from the conventional protein kinases and instead resembles the tertiary structure of metabolic ATP-grasp kinases [Yamaguchi et al., 2001]. This finding raises intriguing questions regarding substrate specificity, particularly with respect to the hypothesis of Ryazanov et al. [1999] that alpha kinases may tend to phosphorylate residues contained in alpha helices rather than loops, turns or unstructured peptides [Ryazanov et al., 1999]. Other kinases, apparently unrelated to the conventional protein kinases when their primary structure was analyzed, folded in tertiary structures closely related to the ser/thr and tyr kinases [Eichinger et al., 1996; Hon et al., 1997; Steinbacher et al., 1999]. These atypical kinases, however, are isolated examples and, unlike alpha-kinases, have not generated a group of growing number of members to the date.

MHCK B, a novel alpha-kinase from *D. discoideum*, participates in the physiological regulation of myosin II assembly raising interesting questions regarding a threonine-based signaling network controlling cortical myosin II assembly and disassembly. The purified full-length enzyme phosphorylates intact myosin II readily, driving filament disassembly in vitro. The stoichiometry of myosin phosphorylation and the kinetic parameters of MHCK B are comparable to those of MHCK A, suggesting same substrate specificity for both kinases. Although MHCK B shares with MHCK A the property of autophosphorylation in vitro, in our analysis this autophosphorylation did not have a significant effect on the biochemical activity of the enzyme. This behavior differs markedly from MHCK A, where autophosphorylation induces an increase in the V_{max} of the enzyme of at least 50-fold [Medley et al., 1990]. This disparity in enzyme regulation suggests that MHCK B may have distinct modes of regulation relative to MHCK A. Further studies are needed to determine the specific signaling mechanisms that regulate MHCK B activity. MHCK B autophosphorylation could conceivably modify the binding of MHCK B to a regulatory complex and trigger a positive feedback mechanism of regulation. This alternative explanation is consistent with the lack of full correlation between the levels of overexpression and the degree of cor-

tical myosin II disassembly observed in the Ax2/MHCK B^{OE} cells. Detailed molecular analysis of the autophosphorylation sites would clarify the role of autophosphorylation on the activity of MHCK B.

MHCK B overexpression results in defective cell division and partical multinucleation when cells are in suspension culture, a characteristic phenotype of myosin II null cells [De Lozanne and Spudich, 1987]. This overexpression phenotype shows that MHCK B is capable of altering phosphorylation and disassembly of myosin II in vivo. Furthermore, this suspension growth defect is rescued by co-expression of phosphorylation-resistant 3× ALA myosin, which highlights the relevance of these key residues on controlling myosin II function.

Analysis of myosin assembly into Triton-X 100-insoluble cytoskeleton fractions further suggests an in vivo role for this enzyme in the control of myosin II assembly. Ax2/MHCK B^{OE} cells show a modest but statistically significant decrease in myosin II filaments, integral elements of the Triton-insoluble cytoskeletons. This decrease in the amount of myosin II filaments in the cytoskeletons is consistent with the hypothesis that MHCK B overexpression results in MHC hyperphosphorylation thus reducing the in vivo filament assembly levels. Although this reduction in the cortical myosin filaments assembled is statistically significant, it is not as high as it might be expected from a 75-fold overexpression. We consider this an indication of the existence of some additional control mechanism limiting MHCK B activity upon overexpression.

Myosin II filaments overassemble significantly in the *mhkB* null cell lines, confirming a physiological role for MHCK B in filament assembly control. The results obtained from the *mhkB* null mutant are consistent with the hypothesis that loss of *mhkB* results in a reduction in MHC phosphorylation levels in vivo, but contrast with the results obtained from the overexpressing cell lines. Once again this lack of correlation between the two assays hints the existence of an additional element involved in the control of MHCK B activity. Notably, the level of overassembly observed here with *mhkB* disruption is very similar to that reported previously in cells disrupted for MHCK A [Kolman et al., 1996]. The partial nature of the myosin II overassembly in *mhkB* null cells and that observed earlier for *mhkA* null cells suggests that these enzymes work together to maintain the correct proportion of assembled versus monomeric myosin within the cell. These studies, however, do not rule out the possibility of these enzymes influencing each other. Additional studies using *mhkA* null cell lines and double *mhkA–mhkB* knocked out mutant may shed light on the interesting possibility of a threonine phosphorylation-based signaling cascade controlling myosin II function. Our recent identification of differential cellular localization patterns for MHCK A, MHCK B, and MHCK C further support the hypothesis of differential regulation of activity for each of these enzymes [Liang et al., 2002].

The studies presented here provide the first analysis to date of the in vivo roles of MHCK B and the first biochemical analysis performed with the full-length enzyme. This work provides strong support for the hypothesis that MHCK B contributes to the physiological regulation of myosin II assembly in *Dictyostelium*. A series of earlier studies have identified Akt/PKB [Chung et al., 2001], a PAK family kinase [Chung and Firtel, 1999], and cGMP signaling (reviewed in Goldberg et al. [2002]) as upstream regulators of myosin II localization in *Dictyostelium*, but direct biochemical links have not been identified between these enzymes and MHCK B or any of the other MHCKs in this system. Further biochemical and cellular studies are needed to clarify how these upstream signaling pathways modulate myosin II phosphorylation and filament assembly. Based upon the analysis presented here, and upon recent analysis MHCK C [Liang et al., 2002], we suggest that members of this family of enzymes function in co-ordination with each other to regulate myosin II assembly and localization during actomyosin-based cell motility events. Further gene targeting studies and in vivo localization analysis are needed to resolve specific roles of each enzyme.

REFERENCES

Adachi H, Hasebe T, Yoshinaga K, Ohta T, Sutoh K. 1994. Isolation of *Dictyostelium discoideum* cytokinesis mutants by restriction enzyme-mediated integration of the blasticidin S resistance marker. Biochem Biophys Res Commun 205:1808–1814.

Berlot CH, Devreotes PN, Spudich JA. 1987. Chemoattractant-elicited increases in *Dictyostelium* myosin phosphorylation are due to changes in myosin localization and increases in kinase activity. J Biol Chem 262:3918–3926.

Chung CY, Firtel RA. 1999. PAKa, a putative PAK family member, is required for cytokinesis and the regulation

of the cytoskeleton in *Dictyostelium discoideum* cells during chemotaxis. J Cell Biol 147:559–576.

Chung CY, Potikyan G, Firtel RA. 2001. Control of cell polarity and chemotaxis by Akt/PKB and PI3 kinase through the regulation of PAKa. Mol Cell 7:937–947.

Clancy CE, Mendoza MG, Naismith TV, Kolman MF, Egelhoff TT. 1997. Identification of a protein kinase from *Dictyostelium* with homology to the novel catalytic domain of myosin heavy chain kinase A. J Biol Chem 272:11812–11815.

Côté GP, Luo X, Murphy MB, Egelhoff TT. 1997. Mapping of the novel protein kinase catalytic domain of *Dictyostelium* myosin II heavy chain kinase A. J Biol Chem 272: 6846–6849.

De la Roche MA, Côté GP. 2001. Regulation of *Dictyostelium* myosin I and II. Biochim Biophys Acta 1525:245–261.

De Lozanne A, Spudich JA. 1987. Disruption of the *Dictyostelium* myosin heavy chain gene by homologous recombination. Science 236:1086–1091.

Egelhoff TT, Côté GP. 1999. Myosin heavy chain kinases. In: Kreis T, Vale R, editors. Guidebook to the cytoskeletal and motor proteins. Oxford University Press. Oxford, England. pp 460–464.

Egelhoff TT, Lee RJ, Spudich JA. 1993. *Dictyostelium* myosin heavy chain phosphorylation sites regulate myosin filament assembly and localization in vivo. Cell 75: 363–371.

Eichinger L, Bomblies L, Vandekerckhove J, Schleicher M, Gettemans J. 1996. A novel type of protein kinase phosphorylates actin in the actin–fragmin complex. EMBO J 15:5547–5556.

Goldberg JM, Bosgraaf L, Van Haastert PJ, Smith JL. 2002. Identification of four candidate cGMP targets in *Dictyostelium*. Proc Natl Acad Sci USA 99:6749–6754.

Hadwiger JA, Firtel RA. 1992. Analysis of G alpha 4, a G-protein subunit required for multicellular development in *Dictyostelium*. Genes Dev 6:38–49.

Hanks SK, Quinn AM. 1991. Protein kinase catalytic domain sequence database: Identification of conserved features of primary structure and classification of family members. Methods Enzymol 200:38–62.

Hon WC, McKay GA, Thompson PR, Sweet RM, Yang DS, Wright GD, Berghuis AM. 1997. Structure of an enzyme required for aminoglycoside antibiotic resistance reveals homology to eukaryotic protein kinases. Cell 89:887–895.

Kolman MF, Egelhoff TT. 1997. *Dictyostelium* myosin heavy chain kinase A subdomains. Coiled-coil and WD repeat roles in oligomerization and substrate targeting. J Biol Chem 272:16904–16910.

Kolman MF, Futey LM, Egelhoff TT. 1996. *Dictyostelium* myosin heavy chain kinase A regulates myosin localization during growth and development. J Cell Biol 132: 101–109.

Levi S, Polyakov M, Egelhoff TT. 2000. Green fluorescent protein and epitope tag fusion vectors for *Dictyostelium discoideum*. Plasmid 44:231–238.

Liang W, Licate L, Warrick H, Spudich J, Egelhoff T. 2002. Differential localization in cells of myosin II heavy chain kinases during cytokinesis and polarized migration. BMC Cell Biol 3:19.

Luo X, Crawley SW, Steimle PA, Egelhoff TT, Côté GP. 2001. Specific phosphorylation of threonine by the *Dictyostelium* myosin ii heavy chain kinase family. J Biol Chem 276:17836–17843.

Medley QG, Gariepy J, Côté GP. 1990. *Dictyostelium* myosin II heavy-chain kinase A is activated by autophosphorylation: Studies with *Dictyostelium* myosin II and synthetic peptides. Biochemistry 29:8992–8997.

Medley QG, Lee SF, Côté GP. 1991. Purification and characterization of myosin II heavy chain kinase A from *Dictyostelium*. Methods Enzymol 196:23–34.

Murphy MB, Egelhoff TT. 1999. Biochemical characterization of a *Dictyostelium* myosin II heavy chain phosphatase that promotes bipolar filament assembly. Eur J Biochem 264:582–590.

Pavur KS, Petrov AN, Ryazanov AG. 2000. Mapping the functional domains of elongation factor-2 kinase [in process citation]. Biochemistry 39:12216–12224.

Pinna LA, Ruzzene M. 1996. How do protein kinases recognize their substrates? Biochim Biophys Acta 1314: 191–225.

Price NT, Redpath NT, Severinov KV, Campbell DG, Russell JM, Proud CG. 1991. Identification of the phosphorylation sites in elongation factor-2 from rabbit reticulocytes. FEBS Lett 282:253–258.

Redpath NT, Price NT, Severinov KV, Proud CG. 1993. Regulation of elongation factor-2 by multisite phosphorylation. Eur J Biochem 213:689–699.

Runnels LW, Yue L, Clapham DE. 2001. TRP-PLIK, a bifunctional protein with kinase and ion channel activities. Science 291:1043–1047.

Ryazanov AG. 2002. Elongation factor-2 kinase and its newly discovered relatives. FEBS Lett 514:26–29.

Ryazanov AG, Ward MD, Mendola CE, Pavur KS, Dorovkov MV, Wiedmann M, Erdjument-Bromage H, Tempst P, Parmer TG, Prostko CR, Germino FJ, Hait WN. 1997. Identification of a new class of protein kinases represented by eukaryotic elongation factor-2 kinase. Proc Natl Acad Sci USA 94:4884–4889.

Ryazanov AG, Pavur KS, Dorovkov MV. 1999. Alpha-kinases: A new class of protein kinases with a novel catalytic domain [letter]. Curr Biol 9:R43–R45.

Spudich A. 1987. Isolation of the actin cytoskeleton from amoeboid cells of *Dictyostelium*. Methods Cell Biol 28: 209–214.

Steimle PA, Naismith T, Licate L, Egelhoff TT. 2001a. WD repeat domains target *Dictyostelium* myosin heavy chain kinases by binding directly to myosin filaments. J Biol Chem 276:6853–6860.

Steimle PA, Yumura S, Côté GP, Medley QG, Polyakov MV, Leppert B, Egelhoff TT. 2001b. Recruitment of a myosin heavy chain kinase to actin-rich protrusions in *Dictyostelium*. Curr Biol 11:708–713.

Steinbacher S, Hof P, Eichinger L, Schleicher M, Gettemans J, Vandekerckhove J, Huber R, Benz J. 1999. The crystal structure of the *Physarum polycephalum* actin-fragmin kinase: An atypical protein kinase with a specialized substrate-binding domain. EMBO J 18:2923–2929.

Sussman M. 1987. Cultivation and synchronous morphogenesis of *Dictyostelium* under controlled experimental conditions. In: Spudich JA, editor. *Dictyostelium discoideum*: Molecular approaches to cell biology. Academic Press. Orlando, FL. pp 9–29.

Yamaguchi H, Matsushita M, Nairn AC, Kuriyan J. 2001. Crystal structure of the atypical protein kinase domain of a TRP channel with phosphotransferase activity. Mol Cell 7:1047–1057.