Recruitment of a myosin heavy chain kinase to actin-rich protrusions in *Dictyostelium*

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Abstract:

Nonmuscle myosin II plays fundamental roles in cell body translocation during migration and is typically depleted or absent from actin-based cell protrusions such as lamellipodia, but the mechanisms preventing myosin II assembly in such structures have not been identified [1], [2] and [3]. In *Dictyostelium discoideum*, myosin II filament assembly is controlled primarily through myosin heavy chain (MHC) phosphorylation. The phosphorylation of sites in the myosin tail domain by myosin heavy chain kinase A (MHCK A) drives the disassembly of myosin II filaments in vitro and in vivo [4]. To better understand the cellular regulation of MHCK A activity, and thus the regulation of myosin II filament assembly, we studied the in vivo localization of native and green fluorescent protein (GFP)-tagged MHCK A. MHCK A redistributes from the cytosol to the cell cortex in response to stimulation of *Dictyostelium* cells with chemoattractant in an F-actin-dependent manner. During chemotaxis, random migration, and phagocytic/endocytic events, MHCK A is recruited preferentially to actin-rich leading-edge extensions. Given the ability of MHCK A to disassemble myosin II filaments, this localization may represent a fundamental mechanism for disassembling myosin II filaments and preventing localized filament assembly at sites of actin-based protrusion.

Article:

Results and discussion

Myosin heavy chain kinase A (MHCK A) is the prototypic member of a novel family of protein kinases [5], [6] and [7] and is a major regulator of myosin II assembly in *Dictyostelium* [8] and [9]. MHCK A has a domain structure consisting of an amino-terminal 70 kDa coiled-coil oligomerization domain, a central catalytic domain unrelated to conventional eukaryotic protein kinases, and a carboxy-terminal WD repeat domain. The WD repeat domain displays myosin II filament binding activity in vitro and targets kinase activity to this substrate [10].

MHCK A localizes to actin-rich protrusions

During the aggregation stage of development, *Dictyostelium* responds to a gradient of the extracellular chemoattractant cAMP by becoming highly polarized and migrating into cellular streams that move toward the source of chemoattractant [3]. Immunohistochemical localization of native MHCK A in streaming *Dictyostelium* Ax2 cells revealed an enrichment of MHCK A at the actin-rich leading edge of cells (Figure 1a). The same anterior localization during streaming was observed for epitope-tagged MHCK A (data not shown). Dynamic imaging of live cells expressing a GFP-tagged MHCK A (GFP-MHCK A) fusion revealed a similar localization pattern, with GFP-MHCK A frequently enriched in protrusions formed at the cell anterior (Figure 1b). Further observations of cells expressing FLAG-MHCK A and GFP-MHCK A (not shown) revealed that MHCK A also localizes to lateral cellular extensions of nonpolarized cells.

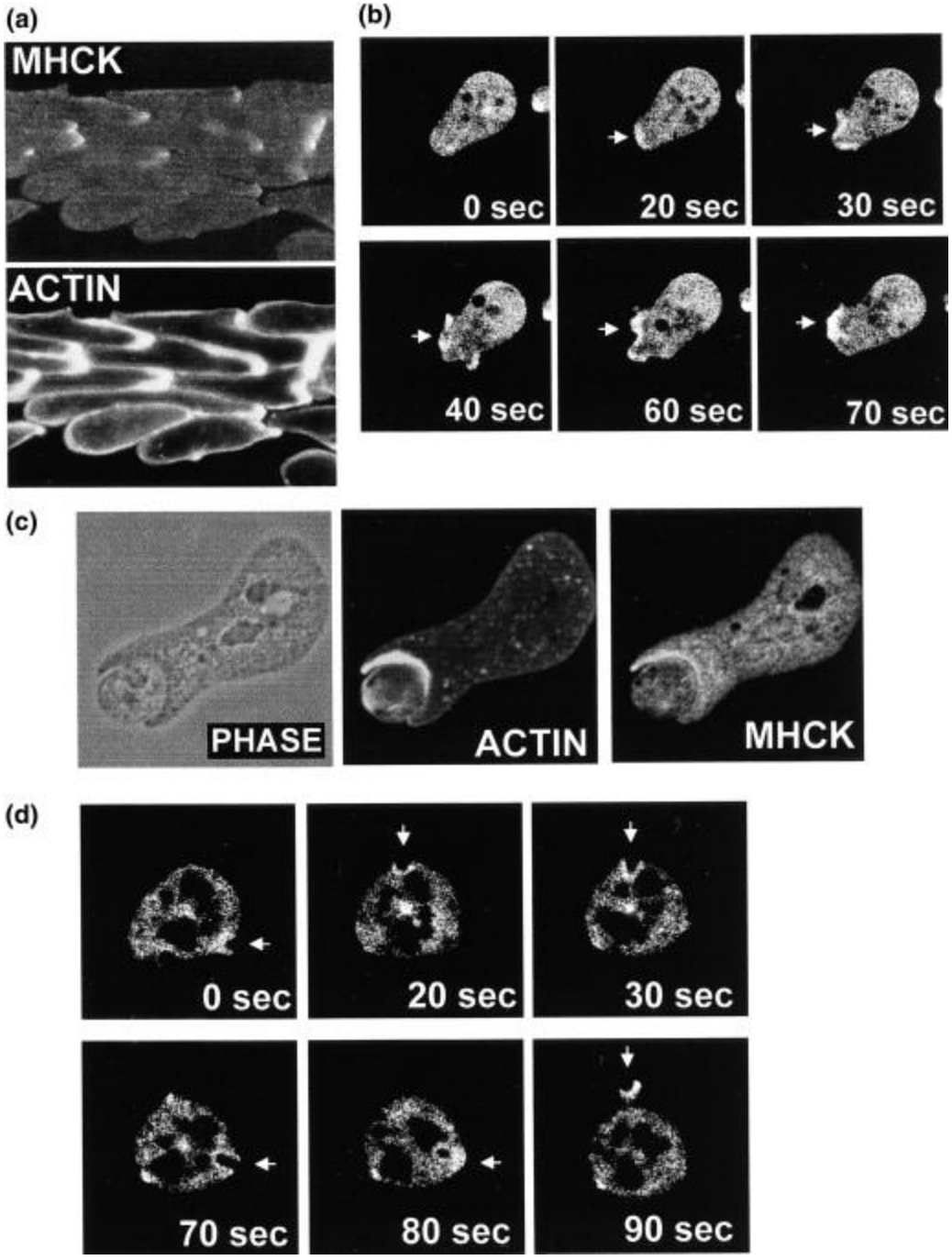

Figure 1. MHCK A is enriched in actin-rich extensions. **(a)** Immunostains of fixed Ax2 cells in chemotactic aggregation streams reveals the enrichment of MHCK A at the leading edge. Staining was performed with a monoclonal anti-MHCK A antibody and fluorescein-labeled secondary antibody. **(b)** GFP-MHCK A fusion protein displays enrichment in actin-rich protrusions. Images were collected at indicated intervals via confocal microscopy. **(c)** FLAG-tagged MHCK A was localized to phagocytic cups via indirect immunofluorescence with an anti-FLAG epitope antibody and a fluorescein-labeled secondary antibody. **(d)** GFP-tagged MHCK A displays dynamic localization to macropinocytic cups. Arrows indicate two macropinocytic cups that formed in one cell during the course of confocal-image collection. See the supplementary material available with this article on the internet for movies corresponding to panels (b) and (d)

The uptake of nutrients from the environment involves many of the same cytoskeletal changes that are known to occur at the leading edge of a migrating cell. In *Dictyostelium*, this involves the formation of actin-rich "crowns" at sites of macropinocytosis and phagocytosis. FLAG-MHCK A becomes enriched in phagocytic cups, and this observation implies a role for MHCK A in the disassembly of myosin II at these sites (Figure 1c). Imaging of live cells expressing GFP-MHCK A revealed that MHCK A also associates transiently with macropinocytic cups that form spontaneously on *Dictyostelium* cells in culture (Figure 1d).

Cyclic AMP induces translocation of MHCK A to the cell cortex

The enrichment of MHCK A at the leading edge of chemotaxing cells suggests that cAMP-activated signaling pathways may regulate MHCK A translocation to actin-rich cortical structures. To test this possibility, we assessed the effect of cAMP stimulation on native MHCK A in Ax2 cells by using conventional immunomicroscopy (Figure 2a). Prior to stimulation (0 s panel), some MHCK A enrichment in pseudopodia can be detected, but the bulk of the MHCK A signal is diffuse and cytosolic. Upon cAMP stimulation, rapid further enrichment of MHCK A in pseudopods is detectable (8 s panel). This enrichment is first apparent in preformed pseudopodia, but it is later detectable throughout the cell cortex (30 s panel). Similar behavior was observed in live cells via the imaging of GFP-MHCK A in cells stimulated with chemoattractant (Figure 2b). GFP-MHCK A displays a relatively diffuse distribution within the cell initially but becomes detectably enriched in the cell cortex as early as 15 s after cAMP stimulation. The kinetics of GFP-MHCK A recruitment are roughly similar to those observed for the recruitment of GFP-myosin II (data not shown). In cells expressing GFP-MHCK A, brighter cells sometimes displayed intensely fluorescent spots that are likely to have been nonphysiological aggregates of the overexpressed GFP-MHCK A. Similar dots of aggregated myosin II result upon forced overexpression of the myosin heavy chain gene [4]. These dots were never observed in wild-type cells immunostained with MHCK A-specific antibodies.

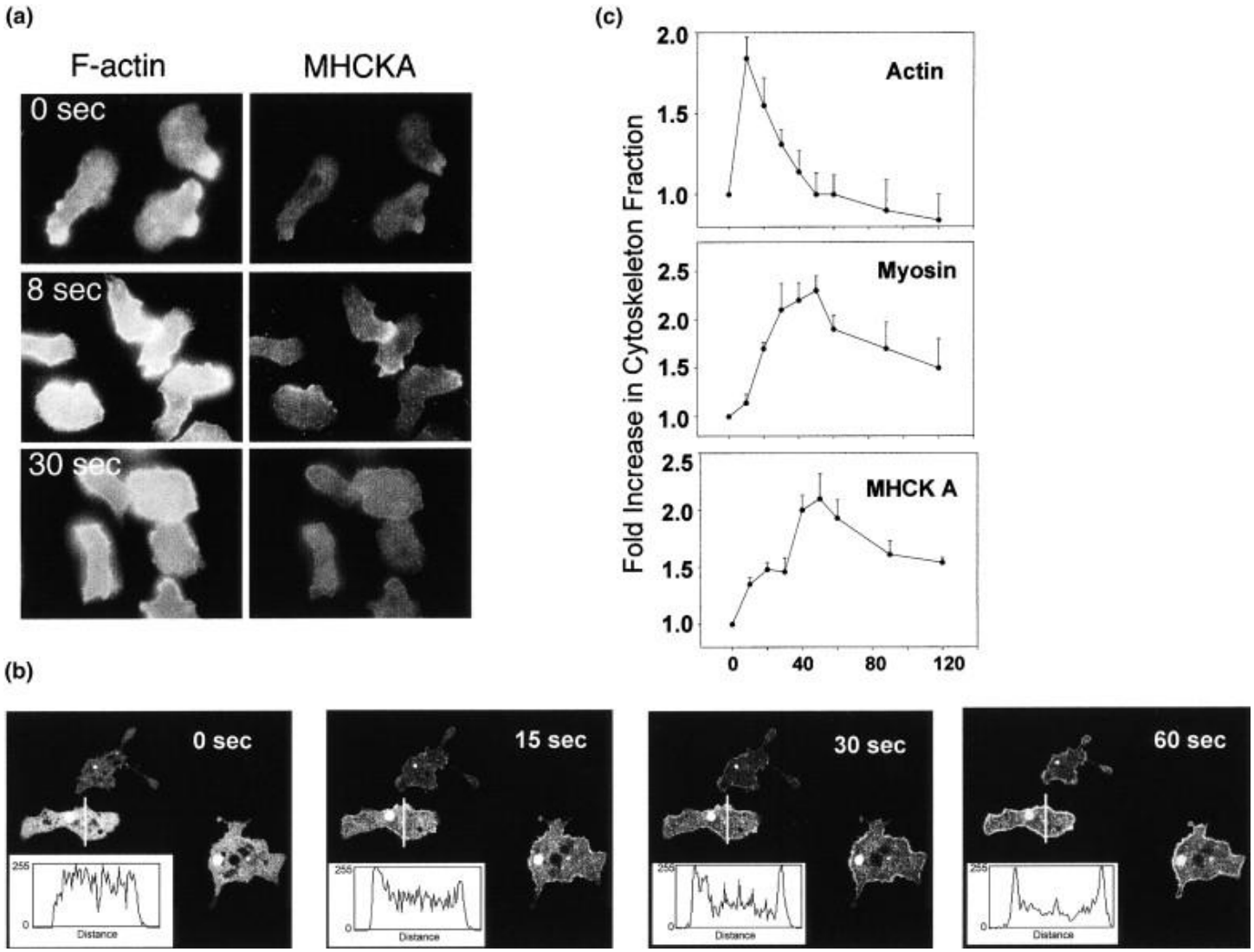

Figure 2. Cyclic AMP induces translocation of MHCK A. **(a)** Chemoattractant stimulation of wild-type Ax2 cells induces the recruitment of native MHCK A to the cell cortex. Examples are shown of cells fixed without cAMP stimulation (0 s) or following cAMP stimulation (8 s, 30 s). Samples were fixed and stained with tetramethylrhodamine-labeled phalloidin (left) and an anti-MHCK A monoclonal antibody (right). **(b)** GFP-MHCK A fusion protein translocation to the cell cortex in response to chemoattractant stimulation. Chemoattractant-responsive cells were treated with 100 μM cAMP and GFP fluorescence images collected at the

indicated time points. Insert shows quantification of fluorescence intensity across the transect indicated by the white bar. **(c)** MHCK A is recruited into the Triton X-100-resistant cytoskeleton in response to chemoattractant stimulation. Cells were stimulated with cAMP (100 μM), and cytoskeletal ghosts were collected at the indicated number of seconds following stimulation. Cytoskeletal fractions were subjected to SDS-PAGE. Actin and myosin recruitment were assessed with Coomassie stain, and MHCK A recruitment was assessed by a Western blot, quantified via densitometry. Actin and myosin II were recruited into the cytoskeletal fraction, with peaks at approximately 20 s and 50 s, respectively. MHCK A displayed a reproducible biphasic recruitment, with a first peak initiated by 10–20 s and a second, larger peak at approximately 40–50 s. Error bars represent standard error of the mean, n = 6

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We performed parallel studies to assess the recruitment of MHCK A into the Triton X-100-insoluble (cytoskeleton-enriched) fraction of cells. Previous studies using this method have established that cells exposed to uniform, saturating levels of cAMP display dramatic and transient increases in both actin polymerization and myosin II filament assembly [11] and [12]. The increase in cortically localized MHCK A observed via microscopy was paralleled by an increase in the amount of MHCK A that was associated with the Triton-resistant cytoskeleton (Figure 2c). As was observed for the cortical localization by immunomicroscopy, this association is rapid (apparent as early as 10 s) and may reflect a stable association of MHCK A with some cytoskeletal element that occurs in response to chemoattractant stimulation.

MHCK A translocation requires F-actin but not myosin II

Since myosin II is the substrate for MHCK A, it seemed possible that cAMP-stimulated recruitment of MHCK A to the cortex might depend on the presence of myosin II in the cell. To test this, we first monitored the effect of cAMP stimulation on the level of MHCK A in cytoskeleton-enriched fractions of MHC null cells. We found that MHCK A in these cells exhibits a similar magnitude of cAMP-stimulated redistribution to Triton-insoluble cytoskeletal pellets as observed for MHCK A expressed in wild-type cells (Figure 3a). Likewise, imaging of live MHC null cells expressing GFP-MHCK A showed typical cortical recruitment of MHCK A in response to cAMP stimulation (Figure 3b, left panels), and this finding demonstrates that myosin II is not required for cAMP-induced translocation of MHCK A.

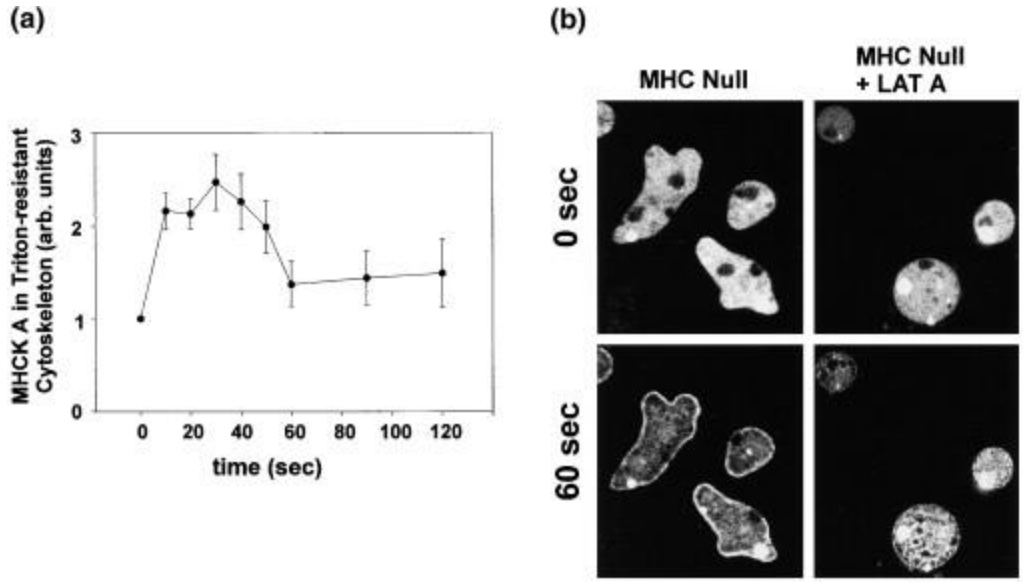

Figure 3. **(a)** Native MHCK A is recruited to the Triton-resistant cytoskeleton in myosin II null cells. Cell stimulation and Western-blot quantification were performed as in Figure 2. **(b)** GFP-MHCK A displays recruitment to the cell cortex in myosin II null cells in response to cAMP stimulation (0 and 60 s time points are shown, left panels). Preincubation of cells for 15 min in latrunculin A blocks localization to the cell cortex in response to cAMP (right panels, 0 and 60 s time points are shown)

To determine if actin filaments are necessary for MHCK A translocation, we treated wild-type (not shown) and myosin II null (Figure 3b, right panels) *Dictyostelium* cells expressing GFP-MHCK A with the drug Latrunculin A (Lat A). In the presence of Lat A, which promotes the disassembly of existing actin filaments and inhibits *de novo* formation of filaments, unstimulated cells round up, and GFP-MHCK A displays a diffuse cytosolic distribution. Upon exposure of these cells to cAMP, GFP-MHCK A becomes more aggregated within the cytoplasm but does not relocalize to the cell cortex (Figure 3b, right panels). Thus, it appears that specific targeting of MHCK A to the cell cortex occurs by a mechanism that requires actin filaments but not myosin II.

A polarized distribution of filamentous actin and myosin II has been observed in many motile cell types. The absence of myosin II-based crosslinking at sites of actin-based protrusion is thought to be important for normal protrusive activity [3] and [13], but to date no mechanisms have been identified that prevent localized myosin II assembly or that can drive localized myosin II disassembly during changes in directionality. The dynamic recruitment of MHCK A to the cell cortex that is reported here, together with its preferential enrichment into actin-rich protrusions, may provide a direct mechanism by which *Dictyostelium* cells prevent myosin II assembly in localized areas where actin assembly processes have been activated to create lamellipodia or related endocytic projections. This in turn may contribute to the establishment of the persistent gradient of cortical myosin II filaments that is observed in chemotaxing *Dictyostelium* cells.

MHCK A is one member of a family of three similar kinases in *Dictyostelium* that include MHCK B [14] and a gene product that has been named MHCK C (GenBank entry #AF079447). While these other gene products are not yet well characterized, current data suggest at least some functional redundancy with MHCK A. MHCK A is expressed during both vegetative growth and multicellular development and thus is a candidate for the regulation of myosin II localization during cytokinesis and contractile ring formation as well as during chemotaxis. A structurally unrelated enzyme, alternatively named DAGK/MHCK [15] or MHC-PKC [16], has previously been reported to participate in the regulation of myosin II assembly during chemotaxis in *Dictyostelium* [16]. Intriguingly, this enzyme contains a highly conserved diacylglycerol kinase domain [15], and this finding suggests a possible role for lipid signaling pathways in the control of myosin II assembly. Further localization and domain analysis of the DAGK/MHCK enzyme are needed to clarify its roles and activities.

A prediction of the model proposed here is that disruption of multiple members of the MHC kinase family should result in defects in extension of lamellipodia and related actin-rich structures. Multiple gene disruptions will be necessary for the full testing of this prediction, but earlier studies with a phosphorylation-resistant "3X ALA" myosin cell line [4] have demonstrated severe defects in lamellipod extension. These 3X ALA cells grossly overassemble myosin II and display an inability to project leading-edge extensions that orient accurately toward a chemoattractant source [13]. The impaired locomotion of these cells supports the hypothesis that correctly localized myosin II assembly control is necessary for proper chemotaxis and cell migration. Substantial understanding has emerged in recent years concerning the regulation and mechanics of actin polymerization during lamellipodial extension, particularly with respect to the regulation of the Arp2/3 complex at the leading edges of polarized cells [17]. However, the mechanisms controlling overall polarization and posterior myosin II filament assembly are not understood. In addition to the well-characterized Arp2/3 complex, a number of other proteins such as coronin, myosin Is, and CRAC have been localized to the leading edge of polarized cells [18], [19], [20] and [21], but in most cases the biochemical roles of these proteins at the anterior end are not well understood. In contrast, the localization of MHCK A to anterior actin-rich protrusions is noteworthy in that this enzyme is known to be a potent regulator of myosin II filament assembly. Its localized anterior recruitment may be a mechanism that helps establish the gradient of myosin II filaments observed in polarized cells [2]. This localization may also contribute to the dynamic reorganization of myosin II localization observed during changes in directionality and pseudopod retraction [13] and [22].

Could similar MHC phosphorylation mechanisms regulate nonmuscle myosin II assembly cycling in vertebrate systems? Studies in mammalian systems have demonstrated MHC phosphorylation in vivo in a variety of settings of stimulated cytoskeletal reorganization [23], [24] and [25]. In vitro myosin II tail fragment disassembly via phosphorylation of mapped PKC and casein kinase target sites near the tip of the myosin II tail has also been demonstrated [26]. We suggest that the mechanisms for the control of myosin II assembly in *Dictyostelium* may have functional parallels in mammalian systems. Further in vivo exploration of this possibility is needed.

Supplementary material

A supplementary figure, three supplementary movies, and supplementary materials and methods are available with the electronic version of this article at www://images.cellpress.com/supmat/supmatin.htm.

Acknowledgements

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References

1 T.M. Svitkina, A.B. Verkhovsky, K.M. McQuade and G.G. Borisy, Analysis of the actin-myosin II system in fish epidermal keratocytes: mechanism of cell body translocation, *J Cell Biol* 139 (1997), pp. 397–415.
2 S. Yumura and Y. Fukui, Reversible cyclic AMP-dependent change in distribution of myosin thick filaments in *Dictyostelium*, *Nature* 314 (1985), pp. 194–196.
3 R.A. Firtel and C.Y. Chung, The molecular genetics of chemotaxis: sensing and responding to chemoattractant gradients, *Bioessays* 22 (2000), pp. 603–615.
4 T.T. Egelhoff, R.J. Lee and J.A. Spudich, *Dictyostelium* myosin heavy chain phosphorylation sites regulate myosin filament assembly and localization in vivo, *Cell* 75 (1993), pp. 363–371.
5 G.P. Côté, X. Luo, M.B. Murphy and T.T. Egelhoff, Mapping of the novel protein kinase catalytic domain of *Dictyostelium* myosin II heavy chain kinase A, *J Biol Chem* 272 (1997), pp. 6846–6849.
6 Egelhoff TT, Côté GP: Myosin heavy chain kinases. In *Guidebook to the Cytoskeletal and Motor Proteins*. Edited by Kreis T, Vale R. Oxford University Press; 1999:460-464.
7 A.G. Ryazanov, M.D. Ward, C.E. Mendola, K.S. Pavur, M.V. Dorovkov and M. Wiedmann *et al.*, Identification of a new class of protein kinases represented by eukaryotic elongation factor-2 kinase, *Proc Natl Acad Sci USA* 94 (1997), pp. 4884–4889.

8 M.F. Kolman, L.M. Futey and T.T. Egelhoff, *Dictyostelium* myosin heavy chain kinase A regulates myosin localization during growth and development, *J Cell Biol* 132 (1996), pp. 101–109.
9 M.F. Kolman and T.T. Egelhoff, *Dictyostelium* myosin heavy chain kinase A subdomains. Coiled-coil and WD repeat roles in oligomerization and substrate targeting, *J Biol Chem* 272 (1997), pp. 16904–16910.
10 P.A. Steimle, T. Naismith, L. Licate and T.T. Egelhoff, WD-repeat domains target *Dictyostelium* myosin heavy chain kinases by binding directly to myosin filaments, *J Biol Chem* 276 (2000), pp. 6853–6860.
11 C.H. Berlot, P.N. Devreotes and J.A. Spudich, Chemoattractant-elicited increases in *Dictyostelium* myosin phosphorylation are due to changes in myosin localization and increases in kinase activity, *J Biol Chem* 262 (1987), pp. 3918–3926.
12 S.J. McRobbie and P.C. Newell, Changes in actin associated with the cytoskeleton following chemotactic stimulation of *Dictyostelium discoideum*, *Biochem Biophys Res Commun* 115 (1983), pp. 351–359.
13 J. Stites, D. Wessels, A. Uhl, T. Egelhoff, D. Shutt and D.R. Soll, Phosphorylation of the *Dictyostelium* myosin II heavy chain is necessary for maintaining cellular polarity and suppressing turning during chemotaxis, *Cell Motil Cytoskeleton* 39 (1998), pp. 31–51.
14 C.E. Clancy, M.G. Mendoza, T.V. Naismith, M.F. Kolman and T.T. Egelhoff, Identification of a protein kinase from *Dictyostelium* with homology to the novel catalytic domain of myosin heavy chain kinase A, *J Biol Chem* 272 (1997), pp. 11812–11815.
15 C.D. Thanos and J.U. Bowie, Developmentally expressed myosin heavy-chain kinase possesses a diacylglycerol kinase domain, *Protein Sci* 5 (1996), pp. 782–785.
16 K. Abu-Elneel, M. Karchi and S. Ravid, *Dictyostelium* myosin II is regulated during chemotaxis by a novel protein kinase C, *J Biol Chem* 271 (1996), pp. 977–984.
17 G.G. Borisy and T.M. Svitkina, Actin machinery: pushing the envelope, *Curr Opin Cell Biol* 12 (2000), pp. 104–112.
18 E.L. de Hostos, The coronin family of actin-associated proteins, *Trends Cell Biol* 9 (1999), pp. 345–350.
19 G. Gerisch, R. Albrecht, C. Heizer, S. Hodgkinson and M. Maniak, Chemoattractant-controlled accumulation of coronin at the leading edge of *Dictyostelium* cells monitored using a green fluorescent protein-coronin fusion protein, *Curr Biol* 5 (1995), pp. 1280–1285.
20 E.M. Ostap and T.D. Pollard, Overlapping functions of myosin-I isoforms?, *J Cell Biol* 133 (1996), pp. 221–224. (40)
21 C.A. Parent and P.N. Devreotes, A cell's sense of direction, *Science* 284 (1999), pp. 765–770.
22 S.L. Moores, J.H. Sabry and J.A. Spudich, Myosin dynamics in live *Dictyostelium* cells, *Proc Natl Acad Sci USA* 93 (1996), pp. 443–446.
23 F.N. van Leeuwen, S. van Delft, H.E. Kain, R.A. van der Kammen and J.G. Collard, Rac regulates phosphorylation of the myosin-II heavy chain, actinomyosin disassembly and cell spreading, *Nat Cell Biol* 1 (1999), pp. 242–248.
24 R.I. Ludowyke, I. Peleg, M.A. Beaven and R.S. Adelstein, Antigen-induced secretion of histamine and the phosphorylation of myosin by protein kinase C in rat basophilic leukemia cells, *J Biol Chem* 264 (1989), pp. 12492–12501.
25 K.A. Giuliano, J. Kolega, R.L. DeBiasio and D.L. Taylor, Myosin II phosphorylation and the dynamics of stress fibers in serum-deprived and stimulated fibroblasts, *Mol Biol Cell* 3 (1992), pp. 1037–1048.
26 N. Murakami, L. Kotula and Y.W. Hwang, Two distinct mechanisms for regulation of nonmuscle myosin assembly via the heavy chain: phosphorylation for MIIB and mts 1 binding for MIIA, *Biochemistry* 39 (2000), pp. 11441–11451.