Myosin I phosphorylation is increased by chemotactic stimulation.

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Abstract

Directed cell migration occurs in response to extracellular cues. Following stimulation of a cell with chemoattractant, a significant rearrangement of the actin cytoskeleton is mediated by intracellular signalling pathways and results in polarization of the cell and movement via pseudopod extension. Amoeboid myosin Is play a critical role in regulating pseudopod formation in *Dictyostelium* and their activity is activated by heavy chain phosphorylation. The effect of chemotactic stimulation on the in vivo phosphorylation level of a *Dictyostelium* myosin I, myoB, was tested. The myoB heavy chain is phosphorylated in vivo on serine-322 (the myosin TEDS rule phosphorylation site) in chemotactically-competent cells. The level of myoB phosphorylation increases following stimulation of starving cells with the chemoattractant cAMP. A 3-fold peak increase in the level of phosphorylation is observed at 60 sec following stimulation, a time at which the *Dictyostelium* cell actively extends pseudopodia. These findings suggest that chemotactic stimulation results in increased myoB activity via heavy chain phosphorylation and contributes to the global extension of pseudopodia that occurs prior to polarization and directed motility.
Introduction

The directed movement of cells in response to attractive and repulsive chemical cues is essential for correct development of multicellular organisms and the survival of microorganisms (1). Extracellular chemoattractants bind to G protein-coupled receptors that, in turn, transmit signals to the actomyosin cytoskeleton that generates biased motility (2). The intracellular signals that regulate and coordinate these processes are beginning to be understood but the full pathway from the receptor to the cytoskeleton remains to be elucidated.

*Dictyostelium* has proven to be an invaluable model organism for investigating the molecular basis of chemotaxis. Many, if not all, of the components of the signalling cascade are shared with those found in mammalian cells and the motile response of the cell is indistinguishable from that of a leukocyte (1,2). Stereotypical transient morphological and cytoskeletal changes rapidly occur in *Dictyostelium* amoebae following cAMP stimulation (3). Within the first 20 sec the cell rounds up or "cringes" (4). The cell then actively extends pseudopodia in random directions 60 sec following stimulation. Finally, by 90 - 120 sec, the cell becomes repolarized and moves in the direction of the stimulus (5). These morphological changes are accompanied by alterations in the levels of F-actin and the phosphorylation state of the myosin II heavy chain. Concomitant with the cringe (0 - 20 s) is an increase in total F-actin (with a peak at 10 sec) (5). This is followed by an increase in the level of myosin II heavy chain
phosphorylation (that promotes depolymerization of myosin II thick filaments (6)),
with a peak of phosphorylation at 30 - 40 sec (7,8). Myosin II is also translocated to the
cortex, where it is thought to become more accessible to the heavy chain kinase (9,10),
as well as the actin cross-linking protein, ABP-120 (11). The cortical F-actin levels then
increase again, with a second peak at 60 sec, coincident with active pseudopod
extension (5). This sequence of molecular and morphological events provides a
fundamental framework for understanding how chemotactic stimulation results in
directed cell migration.

A key process in the chemotactic response is the orderly extension of pseudopodia
and work in *Dictyostelium* has revealed that class I myosins play an important role in
regulating their function (12-14). The class I myosins are ubiquitous, they are
expressed in organisms ranging from yeast to man (15). They all share a conserved
motor domain, a light chain binding domain, and a tail region that contains a polybasic
region that directly binds to membranes via electrostatic interactions and also to actin
(15-17). The amoeboid subclass of myosin Is have two additional domains in their tails.
The first is a region rich in the amino acids glycine, serine, and alanine (or glutamate or
serine) that also constitutes an ATP-insensitive actin binding site, and the second is a src
homology 3 (SH3) domain, a known protein-protein interaction domain (18). The in
vitro activity of the class I myosins from lower eukaryotes requires phosphorylation of a
single serine or threonine residue in the motor domain (referred to as the TEDS rule
site; (18,19) by a G-protein regulated myosin I heavy chain kinase (MIHCK) that is a member of the PAK family of kinases (20,21).

Several of the Dictyostelium class I myosins play a role in motility (12,13,22). Two of these myosins, myoA and myoB, have been shown to regulate the number, timing, and placement of pseudopodia (12-14). Consistent with its role in motility, myoB has been shown to be localized to the leading edge of chemotactic cells (23), and its level of expression is significantly increased during aggregation (i.e. when Dictyostelium cells become highly chemotactic) (24). The activity of the amoeboid myosin Is is likely to be strictly regulated in vivo by heavy chain phosphorylation. This has been demonstrated by the finding that mutation of the TEDS rule site at residue 332 from serine to alanine renders this motor inactive in vivo (25-28). Understanding the basis of myosin I functions in vivo requires the identification of the signals and conditions that change its phosphorylation state. While recent progress has been made in the identification and characterization of MIHCKs in both Acanthamoeba and Dictyostelium, virtually nothing is known about the kinase that acts on Dictyostelium myoB, an important regulator of pseudopod formation in Dictyostelium (12,20,21,29,30). The ability to synchronize populations of Dictyostelium during chemotactic stimulation was, therefore, exploited to directly examine changes in the levels of myoB heavy chain phosphorylation in vivo to determine if increased myoB activity is correlated with active pseudopod extension.
Materials and Methods

Maintenance of strains. The Dictyostelium discoideum Ax3 axenic strain and the myoB-strain HTD3-4 (31) were maintained in HL5, a nutrient medium for axenic stains (32), in suspension at 150 rpm. Strains expressing mutant forms of myoB were maintained in HL5 supplemented with 20 µg/ml blasticidin (Calbiochem Chemical Corp., San Diego, CA). The S332A-myoB and myoB/SH3' strains were generated by transformation of the myoB' null strain with an expression plasmid containing altered forms of the myoB gene (27). The S332A-myoB heavy chain carries an alteration in the TEDS rule site, the serine residue at position 332 is changed to alanine. The myoB/SH3' heavy chain is a truncated form of myoB that lacks the C-terminal SH3 domain.

Starvation and cAMP stimulation of Dictyostelium. Prior to all experiments, log-phase cells were collected by centrifugation and washed twice in starvation buffer (20 mM MES pH 6.8, 0.2 mM CaCl₂, 2 mM MgSO₄). Cells were resuspended to a density of 2 - 4 x 10⁷ cell/ml and shaken at 150 rpm for 3.5 hr at room temperature. The onset of starvation initiates the Dictyostelium developmental program and cells prepared in this manner are referred to as aggregation-competent throughout this paper. In some experiments the cells were pulsed with 50 nM cAMP every 6 min for 2.5 hr after the first hour of starvation to ensure initiation of the early developmental program.

Aggregation-competent cells were collected by centrifugation and resuspended in 0.5 ml starvation buffer. A 100 µl sample was taken for the time zero sample and lysed immediately by the addition of an equal volume of SDS (sodium dodecyl sulfate) lysis
buffer (50 mM Tris pH 7.5, 100 mM NaCl, 0.4% SDS, 2 mM EDTA, 50 mM NaF, 50 mM sodium pyrophosphate and fresh 40 mM β-glycerol phosphate, 1 mM sodium orthovanadate, 2 mM ATP, 2 mM DTT (dithiothreitol), and 10 µg/ml each of chymostatin, leupeptin, antipain and pepstatin - CLAP) and stored on ice until further processing. A 1 x 10^{-6} M pulse of cAMP (final concentration) was added to the sample with rapid mixing and 100 µl samples were added to an equal volume of ice-cold SDS lysis buffer at various times point up to 90 sec. The samples were then heated at 100°C for 3 min, cooled to room temperature, and the myoB heavy chain immunoprecipitated as described below.

**Radiolabeling of Dictyostelium proteins.** One ml of cells at a density of 1 - 2 x 10^7 cells/ml were labelled with $^{35}$S-amino acids by incubation at room temperature in starvation buffer containing 0.6 mCi/ml of $^{35}$S-Trans-label (ICN Biomedicals, Irvine, CA) while shaking at 150 rpm for 3.5 hr. Labelled cells were harvested by centrifugation at 510 x g for 5 min and the pellet washed two times with starvation buffer.

Cells were labelled with $^{32}$P to detect phosphorylated proteins by incubating them in the presence of $^{32}$P-orthophosphate during the final stages of starvation. A total of 4 - 8 x 10^7 aggregation-stage cells were collected at 3.5 hr and resuspended in 0.5 ml starvation buffer containing 0.2 to 0.4 mCi/ml $^{32}$P-orthophosphate (ICN Biomedical, Irvine, CA) and 20 mM dithiothreitol (DTT) (8). The cells were then incubated with shaking at 190 rpm for 30 min at room temperature. Some experiments included the
addition of 10 mM caffeine to inhibit extracellular phosphodiesterase following 3 hr of pulsing to suppress spontaneous cAMP oscillations.

**Immunoprecipitation and analysis of phosphorylation levels.** Triton X-100 was added to the cooled cell lysates to a final concentration of 0.2%. Two to three µl of undiluted rabbit polyclonal anti-myoB (31) or rabbit polyclonal anti-mhcA antibody (an antibody specific for the conventional myosin heavy chain, kind gift of Dr. J. Spudich, Stanford University; (8)) were added and the mixtures incubated with gentle shaking for at least 1.5 hr at room temperature. Fifty µl of Protein A-Sepharose bead slurry (Amersham-Pharmacia Biotech, Piscataway, NJ) were added and the sample mixed by gentle shaking at room temperature for 1 hr. In some experiments, the Protein-A-Sepharose beads were pre-cleared by incubation with a myoB- cell lysate prepared in a similar manner for 1 hr and then washed extensively in the lysis buffer. The beads were collected by a brief centrifugation (10 sec) in a microcentrifuge and gently washed 3 times with phosphate-buffered saline followed by 2 washes with lithium buffer (0.5 M LiCl, 0.1 M Tris pH 7.4) to remove non-specifically bound proteins. The washed pellets were then resuspended in 40 µl of 2x SDS-PAGE sample buffer and boiled for 3 min.

The immunoprecipitated gel samples were briefly spun to pellet the beads and equal portions of each supernatant applied to a 7.5% SDS-PAGE gel. Following electrophoresis, the gel was either stained briefly with Coomassie Brilliant Blue R and dried on filter paper or immediately transferred to either PVDF or nitrocellulose
membrane (Millipore, Bedford, MA) (33). The phosphorylated proteins were detected by exposing the dried gel or membrane to Kodak XAR5 film (Eastman-Kodak, Rochester, NY) for 6 - 8 days in the initial experiments. The films were scanned using an Epson ES1200C color scanner (Epson, Pittsburgh, PA) and the amount of signal determined by densitometry of the scanned film using NIH Image 1.54 software. In later experiments, the dried gels or membranes were exposed to a phosphorimage storage screen for 24 to 48 hr and radioactive bands detected using a phosphorimager system (either the Fuji MacBas 1000 phosphorimaging system, Fuji Medical Systems, Stanford, CT or the Molecular Dynamics PhosphorImager 400). Manufacturer's software was used to quantify the signal in each lane. Following autoradiography, the levels of myoB heavy chain on the blotted membranes were determined. The membrane was incubated with the myoB antibody followed by a goat anti-rabbit secondary antibody conjugated to horseradish peroxidase. Detection was performed determined with an ECL kit per manufacturer's instructions (Amersham-Pharmacia Biotech). Multiple exposures of the immunoblot films were taken to ensure that the bands were in the linear range, and these were scanned using an Epson scanner. The amount of protein was determined by densitometry of the scanned film using NIH Image 1.54 software. The relative level of phosphorylation was determined by dividing the amount of phosphorylation determined by autoradiography by the amount of protein at that time point as determined by scans of the western blots.

**Phosphoamino acid determination.** A total of $2 - 4 \times 10^7$ $^{32}$P-labelled aggregation-competent cells in a 2 ml volume were stimulated with chemoattractant by adding 1 x
10^-6 M cAMP, as described above. Sixty seconds after the addition of chemoattractant, the cells were lysed and the myoB heavy chain immunoprecipitated. The entire sample was run on a 6% SDS-PAGE curtain gel, electrotransferred to PVDF and exposed to a phosphorimager screen. The position of the 125 kD phosphorylated myoB heavy chain was confirmed by immunodetection. The major band that correlated with a band of radioactivity was excised from the membrane, rinsed briefly in methanol, then water, and incubated in 6 M HCl under N₂ gas at 100°C for 1 hr in a screw-top vial (34). The hydrolysate was collected and counted by Cherenkov counting to confirm the recovery of ^{32}P. The phosphoamino acids were separated by high voltage electrophoresis on a thin layer chromatography (TLC) plate in parallel with phosphoamino acid standards as described in (35). The pH 1.9 electrophoresis buffer was a 50:156:1794 (v/v/v) mixture of 88% formic acid - glacial acetic acid - dH₂O mixture. Following electrophoresis, the TLC plate was sprayed with ninhydrin to visualize the position of the phosphoamino acid standards and then exposed to a phosphorimager plate overnight.

**In vitro phosphorylation of myoB.** First, a clarified high-speed supernatant was made from aggregation-competent *Dictyostelium* (see above). Cells were collected after 4 hr of starvation and resuspended to a final density of 1 ml per gm cells in 50 mM HEPES, pH 7.5, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 30% sucrose, 1 mM DTT, 5 mM benzamidine-HCl, 40 µg/ml TLCK, 1 mM PMSF, and CLAP. Cells were drop frozen in liquid nitrogen and stored at -80°C. Cells were thawed by dilution to 20% sucrose with ice cold 100 mM HEPES, pH 7.4, 0.5 mM EDTA, 5 mM TAME, 50 µg/ml TLCK, 5 mM
benzamidine-HCl, and CLAP, then lysed with 3 passes through a tight fitting Dounce homogenizer (Wheaton Scientific, Millville, NJ). DTT was added to a final concentration of 1 mM and the lysate clarified by centrifugation at 10,000 x g for 20 min. The supernatant was collected and centrifuged at 50,000 x g for 3 hr at 4°C to obtain supernatant "S2". Ammonium sulfate was added to a final concentration 80% for storage at -20°C. Small samples were dialyzed into HEMK (20 mM HEPES, 1 mM EGTA, 5 mM MgCl$_2$, 50 mM KCl pH 7.4) buffer when needed.

*In vitro* kinase assays of myoB were carried out by incubating 200 µl of S2 plus 1µl of a 1:1:1 mixture of saturated PMSF, 1 M DTT and CLAP with 10 µCi of ATP-$\gamma$-P$_{32}$ for various times at room temperature. Samples also included 10 µl of phosphatase inhibitors (200 mM NaF, 50 mM Na-phosphate, 250 mM β-glycerol phosphate 0.1 nM okadaic acid, 50 µM cypermethrin and deltamethrin) in the reaction mixture. Phosphorylation reactions were stopped by adding lysis buffer without SDS and heating at 100°C for 3 min. The samples were then cooled and the myoB heavy chain was immunoprecipitated as described above. SDS-PAGE samples were applied to a 6% gel, transferred to PVDF membrane, and subjected to autoradiography and immunodetection.
Results

The phosphorylation state of a protein of interest in vivo can be determined by immunoprecipitation from labelled cell lysates. This method also allows one to test for various cellular conditions that could alter the protein’s phosphorylation state. A specific rabbit polyclonal antibody generated against a denatured *Dictyostelium* myoB tail fusion protein (31) was tested for its ability to immunoprecipitate myoB from a cell extract. Because the myoB heavy chain is not a highly abundant protein, cells were removed from nutrient media and starved for 4 hr to increase endogenous levels (24). The cells were labelled with $^{35}$S amino acids and aliquots lysed with detergent, boiled, and incubated with the antibody of interest. The antibody-antigen complex was then isolated using Protein-A Sepharose beads. Following electrophoresis of the samples and autoradiography of the dried gel, a single band at $\approx 125$ kD is observed in immunoprecipitates with the myoB antibody but not when the preimmune sera is used (Fig. 1A, lanes 1 and 2). An antibody that recognizes the myosin II heavy chain was used as a control for the immunoprecipitation method (8) and it was found to precipitate the expected 240 kD band (Fig 1A, lane 3).

The amoeboid myosin Is are phosphorylated in vitro by a specific MIHCK (29,36) and the TEDS rule serine is essential for in vivo function of the lower eukaryotic myosin Is (25-28). Consistent with these findings, it was predicted that the *Dictyostelium* myoB heavy chain would be phosphorylated in vivo. The in vivo phosphorylation of
myoB was examined by incubating aggregation-competent cells with $^{32}$P-orthophosphate followed by immunoprecipitation, electrophoresis, blotting, and autoradiography of the myoB heavy chain. Immunodetection was used to verify the presence of the myoB heavy chain as Coomassie staining of the gel did not always reveal the presence of a prominent band at 125 kD. The immunoprecipitated myoB heavy chain was phosphorylated (Fig. 1B, lane 1). A control experiment that employed a myoB null mutant ($myoB^{-}$) strain was performed in parallel. No 125 kD band was visible either in the immunoblot or in the autoradiogram (Fig. 1B, lane 2), again demonstrating the specificity of the antibody and the immunoprecipitation protocol.

The MIHCKs from *Acanthamoeba* and *Dictyostelium* are present in high-speed supernatants (29,37), suggesting that a significant proportion of these enzymes may be readily solubilized. Since the myoB heavy chain has been shown to be a poor substrate for the *Dictyostelium* MIHCK (29), an extract was tested for a kinase activity that might phosphorylate the myoB heavy chain. A high speed supernatant from aggregation-competent cells enriched for myoB was incubated at room temperature in a reaction buffer containing $^{32}$P-$\gamma$-ATP and a cocktail of phosphatase inhibitors. The myoB heavy chain was immunoprecipitated from the reaction at various times following the addition of ATP, and the level of phosphorylation quantified. A steady increase in the level of myoB phosphorylation was observed over the 30 min time course (Fig. 1C). Little or no phosphorylation was detected in a sample that did not contain phosphatase inhibitors (Fig. 1C).
The identity of the in vivo phosphorylated residue on the myoB heavy chain was determined by phosphoamino acid analysis. The phosphorylated myoB heavy chain was isolated following transfer to PVDF membrane and hydrolyzed by acid. The hydrolysate was then separated by high voltage one-dimensional electrophoresis at pH 1.9. The phosphoamino acid(s) were visualized by autoradiography and the position compared to known standards. Serine was the sole phosphoamino acid detected (Fig. 2). To determine if serine 332 (the TEDS rule site) was the site of phosphorylation, the S332A-myoB heavy chain was immunoprecipitated from cells labelled with $^{32}$P-orthophosphate. Following immunoprecipitation, autoradiography revealed that the S332A-myoB heavy chain was not phosphorylated whilst the control wild type myoB heavy chain was (Fig 3A). Thus, the sole site of myoB heavy chain phosphorylation in vivo is serine 332, the TEDS rule site.

The *Acanthamoeba* and *Dictyostelium* MIHCKs contain a PXXP motif that has the potential to bind to SH3 domains (29,38,39). The amoeboid myosin Is have C-terminal SH3 domains and deletion of the myoB SH3 domain renders this motor non-functional in vivo without altering the localization of this myosin (25,27). One explanation for the inactivity of the myoB/SH3 heavy chain (a truncated myoB heavy chain that lacks the C-terminal SH3 domain) is that the kinase can not bind to the myosin I via its SH3 domain and phosphorylate the TEDS rule site in vivo. The in vivo phosphorylation of the myoB/SH3- heavy chain was examined to test this possibility. Both the wild type and truncated myoB heavy chain are phosphorylated in vivo (Fig. 3B). Therefore, loss of the SH3 domain does not affect the ability of the MIHCK to
phosphorylate the myoB heavy chain in vivo. This result is consistent with a recent analysis of *Acanthamoeba* myosin IC tail mutants demonstrating that deletion of the SH3 domain does not affect either the $K_m$ or $V_{\text{max}}$ for phosphorylation by MIHCK (17).

Cells lacking myoB exhibit defects in pseudopod formation (12), indicating that myosin I activity is required for efficient motility. The effect of chemotactic stimulation on the level of myoB phosphorylation was tested. Aggregation-competent *Dictyostelium* were stimulated with a physiological dose of cAMP as described for the analysis of myosin II phosphorylation in vivo (8). Cells were incubated either in the presence or absence of caffeine (this inhibits extracellular phosphodiesterase activity and prevents the cells from spontaneously generating cAMP oscillations) and $^{32}\text{P}$-orthophosphate and then subjected to a single, physiological, $1 \times 10^{-6}$ M pulse of cAMP. Samples were taken every 20 - 30 sec and the myoB heavy chain immunoprecipitated and analyzed for phosphorylation. The level of myoB heavy chain phosphorylation appeared to increase either in the presence (Fig. 4A) or absence (Fig. 4B) of caffeine, with a peak at 60 sec. This indicates that the increased phosphorylation was not due to an intercellular increase in cAMP but is due to signalling via the cAMP receptor. The relative increase in the level of heavy chain phosphorylation was determined by normalizing the levels of phosphorylation to the amount of myoB in each sample in three independent experiments. The overall level of phosphorylation increased three-fold by 60 sec (Fig. 5) after which time it returned to resting levels.
Discussion

Chemotactic stimulation of Dictyostelium results in a significant increase in myoB heavy chain phosphorylation at the TEDS rule site (Fig. 3 - 5). Higher levels of heavy chain phosphorylation most likely result in increased myosin I activity (18). The observed peak of phosphorylation 60 sec (Fig. 5), a time at which active pseudopod formation takes place, is consistent with a role for myoB in pseudopod formation and cell motility (12). This work reveals that chemotactic signalling through the cAMP receptor results in increases in myosin I activity either by activating MIHCK or by making myosin I more available for phosphorylation.

Phosphorylation of myosin I heavy chains has been reported for Acanthamoeba and brush border myosin Is (40-43). Like Dictyostelium myosin Is, the Acanthamoeba myosin Is are regulated by phosphorylation at the TEDS rule site (18). Immunoelectron microscopy using antibodies specific for the phosphorylated forms of Acanthamoeba myosin IA, IB, and IC revealed that each of these myosin Is are phosphorylated in vivo (43). The fraction of each myosin I that was phosphorylated in vivo varied for each myosin I, but the phosphomyosin Is were concentrated in actively motile regions. The activity of brush border myosin I (BBMI) is, in contrast, regulated by calcium-calmodulin (15). However, the BBMI heavy chain is phosphorylated in cytoskeleton preparations (40,41) and in vitro experiments revealed that BBMI can be phosphorylated by protein kinase C on both serine and threonine when BBMI is
associated with acidic phospholipids (42). The site of phosphorylation resides in the C-terminal tail region but the role of phosphorylation in BBMI function is unknown (42).

A single MIHCK has been identified Dictyostelium (21,29). It is highly homologous to the Acanthamoeba MIHCK and is a member of the PAK family of small G-protein regulated kinases (21,30). The MIHCKs are activated by the non-Dictyostelium small G-proteins Cdc42 and Rac1, autophosphorylation, and acidic phospholipids (20,44). The Dictyostelium G-protein that activates MIHCK in vivo or the exchange and activating factors that act upon it have not yet been identified. However, a number of small G proteins are expressed in aggregation-competent cells and it will be of interest to determine which plays a role in the activation of MIHCK and the signalling pathways that activate it.

The purified Dictyostelium MIHCK does not appear to be capable of phosphorylating the myoB heavy chain (29). Instead, it can phosphorylate the closely related myoD heavy chain (29), a myosin I not known to play a major role in cell motility based on mutant analyses (45). Further underscoring the physiological distinction between myoB and myoD, the overall levels of the myoB heavy chain increase significantly during development (up to 7 fold 8.75 hr after the onset of development) but those of the myoD heavy chain remain unchanged (24). It is, therefore, quite likely that the myoB heavy chain is phosphorylated by a distinct MIHCK, perhaps one that is
developmentally regulated or contains regulatory sequences that respond to signals from the cAMP receptor. The identification of myoB heavy chain kinase activity in cell extracts (Fig. 1C) suggests that a kinase required for activating this myosin I is present during chemotaxis. Purification of this kinase and identification the mechanism of its activation should help to elucidate how stimulation of cells by chemoattractants results in efficient, directed motility.
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Literature Cited

Figures and Legends

Figure 1. Phosphorylation of the myoB heavy chain in vivo and in vitro.  

A. Specificity of the myoB antibody. Antibodies directed against either the myoB heavy chain (lane 1) or the myosin II heavy chain (lane 3) were added to radiolabelled lysates from starved Ax-3 cells and the antibody-antigen complex immunoisolated. myoB preimmune sera was used in a parallel control experiment (lane 2). Samples were subjected to gel electrophoresis and autoradiography. The position of the 125 kD myoB heavy chain and 240 kD myosin II heavy chain are indicated on the right side of the gel.

B. In vivo phosphorylation of myoB. Starved Ax-3 (lane 1) or myoB⁻ (lane 2) cells were harvested and incubated with shaking in the presence of $^{32}$P-orthophosphate. The myoB antibody was incubated with the labelled cell lysates and precipitated with Protein A-Sepharose beads. Samples were subjected to gel electrophoresis, transferred to nitrocellulose and analyzed by autoradiography (phos) and then western blotting with the anti-myoB antibody (α-myoB). Only the region of the gel containing the myoB heavy chain is shown.

C. In vitro phosphorylation of the myoB heavy chain. A high-speed supernatant containing myoB was incubated with $^{32}$P-γ-ATP, myoB immunoprecipitated, and assayed for the level of phosphorylation over time. The 15 min time point marked with "★" is a sample that was incubated for 15 min without the addition of phosphatase inhibitors. Inset, autoradiogram of a myoB immunoprecipitate from a high speed supernatant incubated with $^{32}$P-γ-ATP. Lane 1, 15 min. time point
with phosphatase inhibitors. Lane 2, 15 min. time point without phosphatase inhibitors.

Only the region of the gel containing the myoB heavy chain is shown.
Figure 2. The myoB heavy chain is phosphorylated on serine in vivo. Shown is an autogradiogram of one-dimensional TLC of an acid hydrolysate of the in vivo phosphorylated myoB heavy chain. The positions of phosphoamino acid standards for serine (P-Ser), threonine (P-Thr), and tyrosine (P-Tyr) are indicated. Note that under the conditions used to run the TLC P-Thr and P-Tyr run at the same position. The origin (O) is marked with an X and the large spot near it is likely to be partially hydrolyzed material.
Figure 2.
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Figure 3. Serine 332 is the sole site of phosphorylation on the myoB heavy chain.  A. The S332A-myoB heavy chain is not phosphorylated in vivo. The S332A-myoB heavy chain (lane 2) was immunoprecipitated from radiolabelled extracts and its phosphorylation examined by autoradiography (phos). A control immunoprecipitate from radiolabelled wild type cells is shown for comparison (lane 1). The presence of the myoB heavy chain in each sample was determined by immunoblotting of the same sample (α-myob). B. The myoB/SH3’ heavy chain is phosphorylated in vivo. The myoB heavy/SH3’ chain (lane 2) was immunoprecipitated from radiolabelled extracts and its phosphorylation examined by autoradiography (phos). A control immunoprecipitate from radiolabelled wild type cells is shown for comparison (lane 1). The presence of the myoB heavy chain in each sample was determined by immunoblotting of the same sample (α-myob). Each lane shows only the portions of the gels containing the full-length myoB and S332A myoB heavy chains (125 kD) or the slightly shorter myoB/SH3’ heavy chain (119 kD).
Figure 3.
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A.

\[\text{\(\alpha\)-myoB} \]

\[\text{phos} \]

1 2

B.

\[\text{\(\alpha\)-myoB} \]

\[\text{phos} \]

1 2
Figure 4. Stimulation with chemoattractant results in a transient increase in the level of myoB heavy chain phosphorylation in vivo. Autoradiograms of samples from radiolabeled cells that had been stimulated by 1 x 10^{-6} M cAMP and lysed at various intervals. The myoB heavy chain was immunoprecipitated and the sample subjected to gel electrophoresis, transferred to nitrocellulose and analyzed by autoradiography (phos). Shown are samples from unstimulated cells (U) and from cells 30, 60 and 90 seconds after stimulation. A. Cells incubated without caffeine. B. Cells incubated with 5 mM caffeine.
Figure 4.
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A.

B.
Figure 5. Maximal phosphorylation of the myoB heavy chain occurs 60 sec after stimulation with chemoattractant. The relative amount of myoB heavy chain phosphorylation was determined at various times following stimulation with cAMP. The level of phosphorylation before stimulation (0 sec) is arbitrarily set at 100%. Shown are the results from three independent experiments (□ - Expt. 1; • - Expt. 2; ♦ - Expt. 3).
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