Expression of a Myosin Regulatory Light Chain Phosphorylation Site Mutant Complements the Cytokinesis and Developmental Defects of *Dictyostelium* RMLC Null Cells

Bruce D. Ostrow,* Pengxin Chen,* and Rex L. Chisholm

Department of Cell, Molecular, and Structural Biology, Northwestern University Medical School, Chicago, Illinois 60611

Abstract. In a number of systems phosphorylation of the regulatory light chain (RMLC) of myosin regulates the activity of myosin. In smooth muscle and vertebrate nonmuscle systems RMLC phosphorylation is required for contractile activity. In *Dictyostelium discoideum* phosphorylation of the RMLC regulates both ATPase activity and motor function. We have determined the site of phosphorylation on the *Dictyostelium* RMLC and used site-directed mutagenesis to replace the phosphorylated serine with an alanine. The mutant light chain was then expressed in RMLC null *Dictyostelium* cells (mLCR$^-$) from an actin promoter on an integrating vector. The mutant RMLC was expressed at high levels and associated with the myosin heavy chain. RMLC bearing a ser13ala substitution was not phosphorylated in vitro by purified myosin light chain kinase, nor could phosphate be detected on the mutant RMLC in vivo. The mutant myosin had reduced actin-activated ATPase activity, comparable to fully dephosphorylated myosin. Unexpectedly, expression of the mutant RMLC rescued the primary phenotypic defects of the mlcR$^-$ cells to the same extent as did expression of wild-type RMLC. These results suggest that while phosphorylation of the *Dictyostelium* RMLC appears to be tightly regulated in vivo, it is not essential for myosin-dependent cellular functions.

MYOSIN is responsible for force generation in cortical tension (Clarke and Spudich, 1974; Fukui and Yumura, 1986; Pasternak et al., 1989), cell motility, cytokinesis (Mabuchi and Okuno, 1977; Kiehart et al., 1982; DeLozanne and Spudich, 1987; Knecht and Loomis, 1987; Manstein et al., 1989; Karess et al., 1991; Pollenz et al., 1992), morphogenesis (Knecht and Loomis, 1987; DeLozanne and Spudich, 1987; Pollenz et al., 1992), phagocytosis (Stendahl et al., 1980; Fukui et al., 1990), and membrane receptor capping (Pasternak et al., 1989; Pollenz et al., 1992). In the cellular slime mold *Dictyostelium discoideum*, myosin II is localized in the contractile ring of dividing cells and in the trailing end of locomoting cells (Yumura and Fukui, 1985).

Dictyostelium myosin II is a hexamer composed of two 243-kD heavy chains, two 18-kD regulatory light chains, and two 16-kD essential light chains. The molecule consists of two structural and functional domains: an α-helical coiled-coil rod-like tail responsible for filament assembly (Kuczmarski and Spudich, 1980; DeLozanne and Spudich, 1987; Egelhoff et al., 1991), and a globular head that contains the light chains, actin and ATP-binding sites, and ATPase activity (Côté and Bukiejko, 1987; Warrick and Spudich, 1987; Vibert and Cohen, 1988; Korn and Hammer, 1988; Rayment et al., 1993). Like muscle myosin, *Dictyostelium* myosin II can form bipolar thick filaments in vivo (Yumura and Fukui, 1985; Fukui, 1990) and in vitro (Pasternak et al., 1989).

The activity of *Dictyostelium* myosin is regulated by a myosin-linked process and exhibits properties of assembly and activation distinct from smooth muscle and vertebrate nonmuscle myosin. Assembly of *Dictyostelium* myosin into filaments is regulated by phosphorylation at three sites in the carboxyl-terminal 34-kD of the heavy chain (Côté and Bukiejko, 1987; Vaillancourt et al., 1988; Luck-Vielmetter et al., 1990) which produces a bending of the tail (Claviez et al., 1982; Kuczmarski et al., 1987; Vaillancourt et al., 1988; Lock-Vielmetter et al., 1990; Truong et al., 1992). In contrast, bending of the tail of smooth muscle and vertebrate nonmuscle myosin is regulated by light chain phosphorylation. In these systems phosphorylation of regulatory myosin light chain (RMLC)[1] by a Ca^{2+}-calmodulin dependent MLCK stimulates both assembly and actin-activated ATPase (Suzuki et al., 1978; Somlyo and Somlyo, 1981; Kamm and

* These authors contributed equally to this paper.

Fred Hutchinson's present address is Cancer Research Center, 1124 Columbia St., Seattle, WA 98104.

Address all correspondence to R. L. Chisholm, Dept. of Cell, Molecular, and Structural Biology, Northwestern University Medical School, 303 East Chicago Ave., Chicago, IL 60611. Tel.: (312) 503-4151. Fax: (312) 503-5994.

1. *Abbreviations used in this paper*: MHC, myosin heavy chain; RMLC, regulatory myosin light chain.

Stull, 1985; Ikebe et al., 1987; Trybus and Lowey, 1987), while in *Dictyostelium* phosphorylation of the RMLC regulates only enzymatic activity (Griffith et al., 1987).

The *Dictyostelium* RMLC is phosphorylated in vivo (Kuczmarski and Spudich, 1980) by a specific Ca^{2+}-calmodulin-independent myosin light chain kinase in response to cyclic AMP (Berlot et al., 1985; Griffith et al., 1987; Berlot et al., 1987). RMLC phosphorylation occurs only on serine residues (Berlot et al., 1987). In vitro, RMLC phosphorylation increases the actin-activated Mg^{2+} ATPase activity of myosin (Griffith et al., 1987; Trybus, 1989) and stimulates motor function as assayed by movement of actin filaments in vitro (Griffith et al., 1987). Phosphorylation of purified *Dictyostelium* myosin by cloned *Dictyostelium* MLCK stimulates actin-activated ATPase three- to sixfold and nearly doubles the rate of actin filament movement in vitro (Uyeda and Spudich, 1993; Ruppel et al., 1994). RMLC dephosphorylation restores the activity to a basal level (Kuczmarski and Pagone, 1986; Griffith et al., 1987).

Chen et al. (1994) have produced and characterized RMLC null cell lines, in which the single mlcR gene was disrupted by homologous recombination. The ability to reintroduce light chains into a null background provides the opportunity to assess the in vivo consequences of a variety of RMLC mutations. We have employed this system to investigate the function of RMLC phosphorylation in living cells. We determined the site of RMLC phosphorylation by purified, cloned *Dictyostelium* MLCK (Tan and Spudich, 1991) and used site-directed mutagenesis to eliminate that phosphorylation site. When the mutant RMLC is expressed in *Dictyostelium* it associates with the myosin heavy chain (MHC). Despite its inability to be phosphorylated in vivo, the phosphorylation site mutant rescues the cytokinesis and developmental defects of the RMLC null mutants. Biochemically the mutant myosin exhibits ~30% of the actin activated MgATPase activity of purified wild-type myosin. These results suggest that while phosphorylation of the *Dictyostelium* RMLC appears to be tightly regulated in vivo, it is not essential for completing cytokinesis or morphogenesis.

Materials and Methods

Chemicals were purchased from Sigma Chem. Co. (St. Louis, MO) unless otherwise noted. Potassium iodide, urea, dithiothreitol, glycine, and magnesium chloride were from Research Organics, Inc. (Cleveland, OH). Mercaptoethanol, glycerol, methanol, and sulfuric acid were from Mallinkrodt (Paris, KY). Sodium dodecyl sulfate was from Pierce Chem. Co. (Rockford, IL). Acrylamide and Coomassie were from Bio-Rad Labs. (Richmond, CA). [^{32}P]-ATP (>3,000 Ci/mmol) was from Amersham Corp. (Arlington Heights, IL). Restriction enzymes and Taq polymerase were from Boehringer Mannheim Corp. (Indianapolis, IN).

Cell Culture and Transformations

Dictyostelium discoideum RMLC null cells (strains 43E9, 45E12, and G11D8) have been previously described (Chen et al., 1994). RMLC null cells of all three strains were transfected with 5-μg plasmid DNA by electroporation (Howard et al., 1988) using a BioRad gene pulser at settings 3 μF, 1.3 kV, 25 Ohm, 1 ms time constant. One day after electroporation, media was replaced with HL5 containing 10 μg/ml G418 (Geneticin; Sigma). To establish cell lines, transformants were cloned by plating on *Klebsiella aerogenes* lawns and cells from well-isolated plaques were grown in HL5.

Oligonucleotide-directed Mutagenesis

A full-length RMLC cDNA (Tafuri et al., 1989) in pBluescript (Stratagene Inc., La Jolla, CA) was used as the template for PCR-based mutagenesis. PCR using synthetic primer pair A (5′-GTAAAACGACGGCCAGT-3′) and B (5′-CTACAGAGGCCTCTTCTCTG-3′) produced a 5′ product where the codon for Ser13 (TCA) was changed to Ala (GCC) generating a novel StuI restriction site (see Fig. 2 *a*). PCR using primer pair C (5′-CAGAGAAGAGGCCTCTGTAG-3′) and D (5′-AACAGCTATGACCATG-3′) produced a 3′ product with the same nucleotide substitutions and StuI site. The 5′ and 3′ PCR products were digested with PstI and StuI or StuI and BamHI, respectively, and gel purified. The fragments were ligated together into PstI and BamHI-digested pBluescript vector and sequenced to assure that no additional mutations were introduced by the Taq polymerase. These manipulations restored the reading frame and produced the desired Ser13 to Ala13 codon change. The same strategy was employed to change the codon for Ser14 (TCT) to Ala14 (GCT) using primer pairs A and E (5′-AACTACAGCTGATTCTTCTC-3′) and C and F (5′-GAAGAATCAGCTGTAGTT-3′) which introduce a novel PvuII site. PCR using primer pair C and G (5′-GGGATCCTTATAAATCTTCTTCTGAAATTAATTTTTGTTCTTTTTTACTGAAGAGAG-3′) inserted 30 nucleotides encoding a 10-amino acid epitope of the myc oncogene (EQKLISEEDL, Evan et al., 1985) immediately 5′ to the TAA stop codon of the cDNA.

Vector Construction

Escherichia coli Expression Vectors. PCR was used to remove the untranslated portions of the wild-type RMLC and S13A RMLC cDNAs for subcloning into *E. coli* expression vector pET3d (Novagen Inc., Madison, WI). PCR was done using primer pair H (5′-TGGCCTCAACCAAAAGAAGATTAAAC-3′) and I (5′-ACTTTATTTTTTACTGAAGAGAGTATT-3′) with the wild-type or S13A cDNA as template. The PCR products were subcloned in the sense orientation into the filled-in NdeI site of pET3d, and sequenced. These manipulations inserted the cDNA between a T7 promoter and a T7 terminator and fused 61 nucleotides encoding a six-histidine tag and a thrombin cleavage site to the 5′ end of the RMLC such that a fusion protein was expressed under control of the T7 promoter.

Dictyostelium Expression Vectors. The methionine initiator codon of the *Dictyostelium* actin 15 gene (Knecht et al., 1986) was mutated to a BamHI site and the 270-bp BglII-BamHI promoter fragment was subcloned into pBluescript (Pollenz et al., 1992). A 600-bp BamHI-HindIII (filled-in) fragment of pDNeo2 (gift of A. Noegel, Max Plank Institute, Martinsreid, Germany) containing the *Dictyostelium* actin 8 terminator (McKeown and Firtel, 1981) was subcloned downstream of the mutated actin promoter to produce pBV17. The 2-kb XbaI neomycin phosphotransferase cassette of pDNeo2 was subcloned into the HindIII (filled-in) site of pBV17 to produce pBORP (see Fig. 3 *A*). Wild-type RMLC cDNA, and wild-type and mutant myc-tagged RMLC cDNAs were subcloned in the sense orientation into the BamHI site between the actin promoter and terminator to produce, respectively, pBVN519, pBVN5131, pBVN5115, and pBVN5133.

Protein Preparation

Recombinant RMLC. The pET3d expression constructs were transformed into *E. coli* BL21(DE3). This strain produces T7 RNA polymerase in response to IPTG induction. Bacterial cultures were grown to A_{600} = 0.8, at which time IPTG was added to 0.4 mM and incubated for another 3 h. Cells were sonicated in IMAC-5 (20 mM Tris–HCl, pH 7.9, 0.5 M NaCl, 10% glycerol, 1 mM PMSF, 5 mM imidazole). The lysate was clarified by centrifugation, filtered through a 0.45-μm membrane (Costar Corp., Cambridge, MA), and loaded on a His-Bind resin column (Novagen). His-tagged RMLC was eluted with 60% IMAC-200 (IMAC-5 with imidazole at 200 mM), dialyzed against 20 mM Tris–HCl, pH 8.3, lyophilized, resuspended to a concentration of 2 mg/ml in thrombin digestion buffer (20 mM Tris–HCl, pH 8.4, 0.15 M NaCl, 2.5 mM $CaCl_2$, 10% glycerol), and digested with human thrombin (Novagen) at a enzyme/substrate ratio of 0.5 units/mg.

Myosin. Myosin was purified from *Dictyostelium* cells as described by Pollenz et al. (1992) except ammonium sulphate precipitation was avoided and a Superose 6 gel filtration column (Pharmacia LKB Nuclear, Gaithersburg, MD) was used in place of S-500 gel filtration. The concentration of myosin was determined using bovine serum albumin (Cohn Fraction V) as a standard with the BioRad protein assay.

Actin. Chicken muscle actin was purified from acetone powder according to Pardee and Spudich (1982). On the day of use, 20 mg lyophilized actin was resuspended in Buffer A (2 mM Tris–HCl, pH 8.0, 0.2 mM ATP, 0.2 mM calcium chloride, 0.5 mM β-mercaptoethanol, 0.005% sodium azide), clarified by centrifugation, and the concentration was determined by measuring the A_{290} and dividing by a correction factor of 0.62 (Uyemura et al., 1978).

Metabolic Labeling and Immunoprecipitation of ^{32}P-labeled Myosin

Metabolic labeling was performed using a protocol generously provided by Dr. Janet Smith (Stanford University, Palo Alto, CA). Cells were harvested at 5×10^6 cells/ml, washed and grown in phosphate-free FM medium for 7 h. The cells were then pelleted and resuspended in phosphate-free FM medium at 5×10^7 cells/ml. [^{32}P]orthophosphate (100 μCi) was added to 200-μl aliquots of cells. The cultures were incubated at room temperature for 3 h with end-over-end rotation. Labeled cells were harvested and the myosin heavy chain immunoprecipitated using the 396 MHC-specific monoclonal antibody (generously provided by Dr. G. Gerisch, Max Planck Institute, Martinsreid, Germany) as described by Berlot et al. (1985). Samples were separated by electrophoresis on 15% polyacrylamide gels and transferred to nitrocellulose.

Electrophoretic Analysis

SDS-PAGE and immunoblotting was performed as described in Pollenz et al. (1992) except anti-RMLC mAb 1A2 was reacted at 1:100 dilution overnight, then secondary goat anti-mouse IgG horseradish peroxidase conjugate (Jackson Immunoresearch Labs., Inc., West Grove, PA) was reacted at 1:100,000, and detected by ECL (Amersham).

Urea-glycerol PAGE was performed with a modification of Perrie and Perry (1970). Myosin (1 μg) in storage buffer was boiled in 2× SDS gel sample buffer, cooled, and urea sample buffer added to produce final concentrations of 4.5 M urea, 10.8 mM Tris-HCl, pH 8.6, 86.4 mM glycine, 0.7 mM EDTA, 0.5 mM DTT. The separating gel was 7.5% acrylamide, 0.28% bisacrylamide, 50% glycerol, 20 mM Tris-HCl, pH 8.6, 160 mM glycine. The stacking gel was 3.5% acrylamide, 0.13% bisacrylamide, 50% glycerol, 71 mM Tris-HCl, pH 6.8. The running buffer was 20 mM Tris-HCl, pH 8.6, 160 mM glycine. The samples were run at 4W constant power.

Amino Acid Sequencing

To determine the site of MLCK phosphorylation the sequences of phosphorylated and unphosphorylated RMLC were determined. Thrombin-digested recRMLC (8 μg) was run on 12% SDS-PAGE, transferred to PVDF membrane (Problot; Applied Biosystems Inc., Foster City, CA) for 1.5 hr at 300 mA in 10 mM 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS), pH 11.0, 10% methanol. The membrane was rinsed in Milli-Q water, stained with 0.1% Coomassie Brilliant Blue in 40% methanol, 1% acetic acid, and destained in 50% methanol, 1% acetic acid. The recRMLC band was excised from the membrane, rinsed in 10% methanol, dried, and sequenced on a sequenator (477A; Applied Biosystems) on line with a model 120A PTH analyzer. To determine the sequence of phosphorylated RMLC, thrombin-digested recRMLC was treated with MLCK (5 μg) and subjected to urea-glycerol gel electrophoresis to resolve phosphorylated and unphosphorylated RMLC. Protein was transferred to Problott, visualized by autoradiography, and the band corresponding to phosphorylated recRMLC was excised and sequenced as above.

Enzymatic Assays

ATPase. Purified myosin was treated with 5 mM $CaCl_2$, 10 mM Tris-HCl, pH 8.0, 250 mM KCl, or varying concentrations of rabbit muscle actin in the presence of 10 mM Tris-HCl, pH 7.6, 35 mM KCl, 5 mM $MgCl_2$, 0.2 mM $CaCl_2$ at room temperature. Reactions were initiated by the addition of 1 mM ATP containing ~200,000 cpm/μl [γ^{32}P]-labeled ATP. Released phosphate was measured by the Kind, Otter, and Witman (1986) modification of the method of Pollard and Korn (1973). To each 100-μl reaction 250 μl 1:1 benzene:isobutanol and 50 μl 20 mM silicotungstic acid/10 mM sulfuric acid was added and rapidly mixed. Following the addition of 50 μl 1 mM KH_2PO_4 to serve as carrier phosphate, 40 μl 5% ammonium molybdate in 4 N H_2SO_4 was added and vortexed for 10 s. After allowing the mixture to partition for 5 min, a 50-μl aliquot of the upper organic phase was recovered and counted in Safety-Solve cocktail (Research Products International Corp., Mount Prospect, IL) in a scintillation counter (LS6800; Beckman Instrs., Carlsbad, CA).

MLCK. Recombinant light chain or whole myosin was treated with recombinant MLCK (a generous gift of J. L. Tan, J. Smith, and J. A. Spudich, Stanford University) in 25 mM Triethanolamine, pH 7.5, 5 mM $MgCl_2$, 1 mg/ml BSA, 2 mM ATP, 0.05 μCi/ml [γ^{32}P]ATP, overnight at 4°C (Tan and Spudich, 1991). Reactions were precipitated by addition of equal volume 10% ice-cold trichloroacetic acid, the protein pellet was resuspended in 2× SDS gel sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 10% β-mercaptoethanol, 20% glycerol), sonicated, neutralized with ammonium hydroxide fumes, and boiled before being run on PAGE.

Morphological Analysis

Cells were processed for 4',6'-diamidino 2-phenylindole (DAPI) fluorescence and immunofluorescence according to Pollenz et al. (1992). A mouse cell line producing a mAb (396) directed against the *Dictyostelium* myosin heavy chain (Pagh and Gerisch 1986) was a gift from Professor G. Gerisch. The cell culture supernatant was diluted 1:1 in phosphate buffered saline.

Results

Dictyostelium RMLC Is Phosphorylated on Serine 13 by MLCK

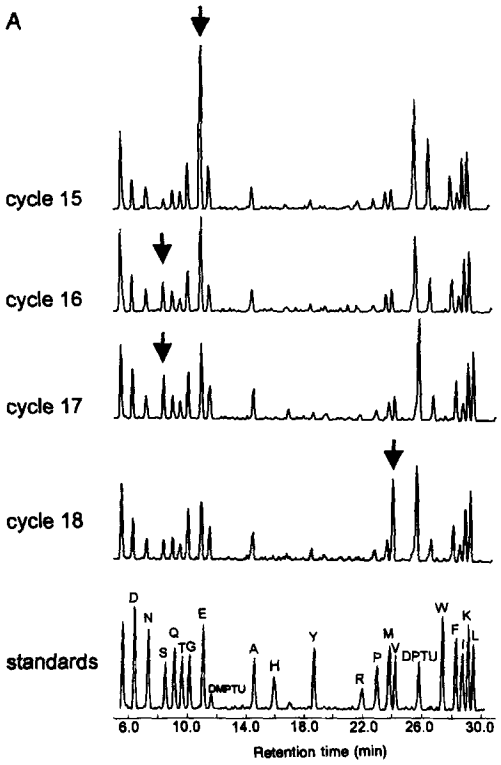
In vivo the *Dictyostelium* RMLC is phosphorylated on serine (Berlot et al., 1987). There are 11 serines in the 161-amino acid polypeptide predicted from the cDNA sequence (Tafuri et al., 1989). Comparison with RMLC phosphorylation sites in other organisms suggested the *Dictyostelium* RMLC phosphorylation site was in the amino terminus of the molecule (Tafuri et al., 1989; Kobayashi et al. 1991). To determine the site of phosphorylation, we sequenced the amino terminus of the RMLC. Because native RMLC purified from *Dictyostelium* cells was blocked at the amino terminus we sequenced recombinant RMLC purified from *E. coli*. Alignment of the sequences of *E. coli*-expressed RMLC phosphorylated in vitro with cloned *Dictyostelium* MLCK (Tan and Spudich, 1991) and unphosphorylated recRMLC was used to identify the phosphorylated residue. PTH-derivatives of phosphoserine are unstable during the acid cleavage step of Edman degradation (Roach and Wang, 1991) and are read as a blank (Ikebe et al., 1987). Purified recRMLC was subjected to 26 cycles of PTH-amino acid sequencing. Unphosphorylated RMLC gave the sequence GSHMASTKRRLNREESSVVLGEEQVA. Fig. 1 *A* shows traces of cycles 15 through 18 of the sequencing reactions performed on unphosphorylated recRMLC. The GSH residues at the NH_2 terminus are derived from the histidine tag used for purification. The remainder of the sequence corresponds to the sequence predicted from the RMLC cDNA (Tafuri et al., 1989). Phosphorylated recRMLC produced the same sequence except that no serine PTH-derivative was detected in cycle 16 (Fig. 1 *B*). The missing residue corresponds to Ser-13 of native RMLC.

Oligonucleotide-directed mutagenesis was employed to convert the codon (TCA) for Ser13 of the cDNA to GCC which codes for a nonphosphorylatable Ala13 (Fig. 2 *A*). This mutation introduces a novel StuI restriction site into the cDNA. As a control for the amino acid substitution we also mutated Ser14 (TCT) to Ala14 (GCT). This mutation introduces a novel PvuII restriction site into the cDNA.

The S13A RMLC cDNA was expressed in *E. coli* as a his-tagged fusion protein. Following purification the expressed RMLC were treated in vitro with MLCK (Fig. 2 *B*) and no phosphorylation was detected. The lowest limit of our detectability is 0.01 mol phosphage/mol RMLC. This result indicates that ser13 is the principal target for the cloned *Dictyostelium* MLCK.

Generation of Mutant Cells

To express the mutant RMLC cDNAs in *Dictyostelium*



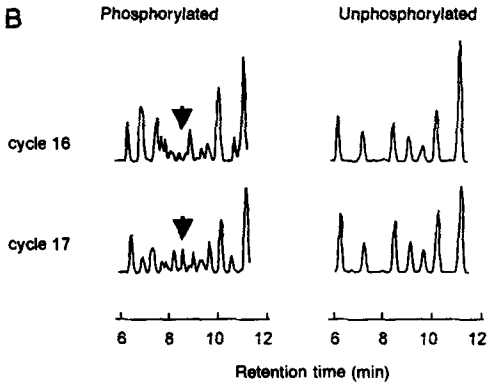


Figure 1. Amino acid sequencing of phosphorylated and unphosphorylated *E. coli*-expressed RMLC indentifies Serine-13 as the site of phosphorylation by MLCK. Recombinant RMLC was treated with MLCK, resolved by urea-glycerol gel electrophoresis and subjected to PTH-amino acid sequencing as described in Materials and Methods. (*A*) PTH-chromatogram profiles of cycles 15 through 18 of unphosphorylated RMLC correspond to the RMLC sequence predicted by the cDNA. (*B*) Detail of PTH-chromatogram profile of cycles 16 and 17 of unphosphorylated (*left*) and phosphorylated (*right*) RMLC. Arrow indicates the position of serine which is absent in cycle 16 of the profile obtained for phosphorylated RMLC, but which can be seen again in cycle 17.

RMLC null cells, we constructed a *Dictyostelium*-expression vector, pBORP (Fig. 3 *A*), which drives expression of inserted cDNAs from the constitutive actin 15 promoter and contains a neomycin resistance gene for selection of transformed cells. The various cDNAs were subcloned into pBORP to produce constructs pBVN5131 (wild type), pBVN5115 (S13A), and pBVN5133 (S14A). To allow detection of the mutant protein when expressed in wild type cells (for experiments not shown), the cDNAs had been tagged at their 3' end with a 10-amino acid epitope for the myc oncogene (Stappenbeck and Green, 1992). Three different RMLC null cell lines were transformed separately with each of the three expression constructs. At least three independent cell lines of each transformation were maintained and analyzed. The myc-tagged wild-type RMLC expressing lines behaved in a similar fashion to the untransformed JH10 parental cell line with respect to development and growth in suspension, except for a slightly slower growth rate which is typical of all of the transformed cell lines.

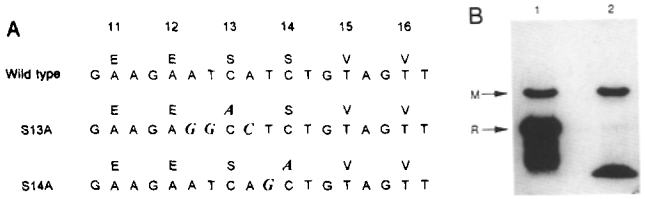


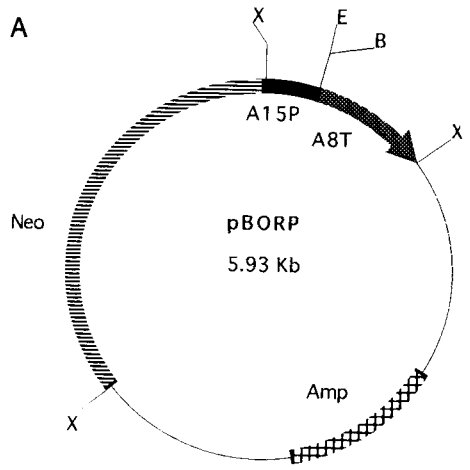
Figure 2. Nucleotide changes in the RMLC cDNA encode an RMLC which cannot be phosphorylated by MLCK. (*A*) The sequence of the RMLC cDNA corresponding to codons 11 through 16 and their respective single-letter amino acid codes are shown for the wild-type (*top*) Serine-13 to Alanine-13 substitution (*middle*) and Serine-14 to Alanine-14 substitution (*bottom*). Differences from wild type are italicized. (*B*) Autoradiograph of 12% SDS-PAGE of wild-type (lane *1*) or S13A (lane 2) recRMLC expressed in *E. coli*, purified, and treated in vitro with MLCK and [γ^{32}P]-ATP. The MLCK is phosphorylated in both samples due to autophosphorylation. *M*, MLCK; *R*, recRMLC.

RNA from cell lines transformed with the constructs showed about 10-fold overexpression of RMLC mRNA (data not shown). RMLC-myc fusion proteins were detected in transformed cell lysates by western immunoblot using an anti-RMLC monoclonal antibody (1A2). The myc-tagged RMLCs migrate with an apparent molecular mass of 19.5 kD (Fig. 3, *B* and *C*). The mutant RMLC copurifies with myosin (Fig. 4 *C*) confirming their association with the MHC.

PCR was performed on DNA purified from transformed cell lines to amplify the RMLC coding sequence. The resulting products were digested with StuI or PvuII (data not shown) to test for the presence of the novel restriction sites introduced by the mutagenesis. PCR products from cells transformed with pBVN5131 (WT) did not cut with StuI or PvuII. Only PCR products from cells transformed with pBVN5115 (S13A) cut with StuI and only PCR products from cells transformed with pBVN5133 (S14A) cut with PvuII. These results confirmed the introduced mutations were maintained in vivo.

S13A RMLC Myosin Is Not Phosphorylated

When myosin purified from S13A and S14A transformed cell lines was treated in vitro with cloned MLCK (Tan and Spudich, 1991), S14A (Fig. 4 *A*, lane 2), but not S13A (lane *1*) RMLC was phosphorylated. This result confirms the identification of S13A as the site of phosphorylation by cloned *Dictyostelium* MLCK. To determine if the S13A and S14A RMLC are phosphorylated in vivo, cells were labeled



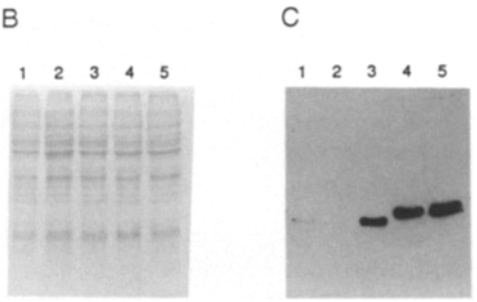


Figure 3. Lysates of RMLC null cells transformed with expression vector pBORP contain RMLC. (*A*) Map of *Dictyostelium* expression vector pBORP. Neo^r = 2-kb neomycin phosphotransferase cassette. A15P = Actin 15 gene promoter where the ATP initiation codon has been mutated to a BamHI site. A8T = Actin 8 gene terminator. Amp^r = ampicillin resistance cassette. Restriction enzyme recognition sites: *X*, XbaI; *B*, BamHI; *E*, EcoRI. 5×10^5 cells of parental strain JH10 (1), null E12 (2), and E12 transformed with wild type (3), S13 A (4), or S14A (5) RMLC cDNAs were lysed and processed for PAGE as described in Materials and Methods. (*B*) Coomassie-stained 12% polyacrylamide gel, and (*C*) corresponding immunoblot reacted with the RMLC-specific monoclonal antibody 1A2. No RMLC is detected in null line E12. S13A and S14A RMLC migrate at apparent molecular mass of 19.5 kD due to extra 10-amino acid COOH-terminal myc epitope tag.

with ^{32}P and myosin was immunoprecipitated using a heavy chain specific antibody. As can be seen in Fig. 4 *B*, the RMLC is labeled on both wild-type (lane *1*) and S14A (lane *3*) mutants, but not on the S13A mutant (lane *2*). Fig. 4 *C* shows the filter in Fig. 4 *B*, probed with an RMLC monoclonal antibody to confirm the presence of RMLC in all samples. The presence of similar levels of RMLC in the immunoprecipitates from all three cell lines indicates that the mutant RMLC associate with the MHC with normal stoichiometry. These results clearly demonstrate that the S13A mutant is not phosphorylated to any significant extent in vivo. The absence of labeling indicates that serine 13 must be the predominant site of RMLC phosphorylation in vivo.

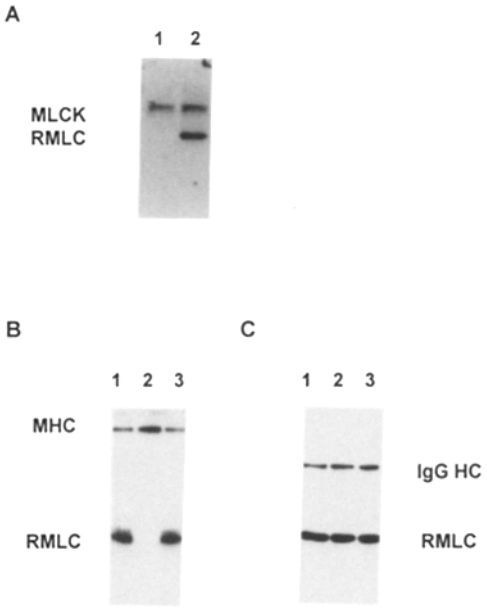


Figure 4. Myosin carrying the S13A RMLC is not phosphorylated in vitro or in vivo. (*A*) Myosin was purified from cells, treated in vitro with MLCK and [γ^{32}P]ATP and analyzed by SDS–polyacrylamide gel electrophoresis. Autoradiogram of in vitro ^{32}P-labeled S13A (lane *1*) or S14A (lane *2*) myosin shows phosphorylation only in S14A RMLC. (*B*) Myosin was immunoprecipitated from ^{32}P-labeled cells expressing wild-type RMLC (lane *1*), S13A RMLC (lane *2*) and S14A RMLC (lane *3*). Following separation by SDS–polyacrylamide gel electrophoresis, transfer to nitrocellulose and autoradiography, MHC phosphorylation can be seen in all three cell lines, and RMLC phosphorylation on wild-type and S14A RMLC, but not S13A RMLC. (*C*) Immunoblot of the filter from B probed with anti-RMLC monoclonal antibody 1A10.

S13A RMLC Myosin Exhibits Basal ATPase Activity

The ATPase activities of myosins purified from the mutant and control cell lines were determined. There was no significant difference between the ATPase activity of myosin from wild-type cells or cells overexpressing wild-type myc-tagged RMLC (data not shown) indicating that neither the G418 drug selection nor the myc tag significantly affects myosin enzymatic activity. S13A myosin had a calcium ATPase activity (V_{max} = 600 nmol Pi/min/mg) comparable to S14A and wild-type myosin. However, S13A myosin had an actin-activated ATPase activity (V_{max} = 20 nmol Pi/min/mg) which was 30% that of S14A (Fig. 5) and wild-type myosin. This value for S13A myosin is the same as that found for phosphatase-treated myosin in Griffith et al. (1987). S14A and wild-type myosin exhibit the same activity as untreated, partially phosphorylated myosin in Griffith et al. (1987).

S13A RMLC Corrects the Developmental Defect of RMLC Null Cell Lines

We next characterized the phenotypes of cell lines expressing the modified RMLCs. The cells are motile and able to undergo cytokinesis on plastic and in shaking culture. When plated on lawns of *Klebsiella aerogenes*, S13A plaques appeared in four days, one day later than S14A plaques. Fruiting bodies developed in most plaques with a morphology and density indistinguishable from wildtype (Fig. 6). Some

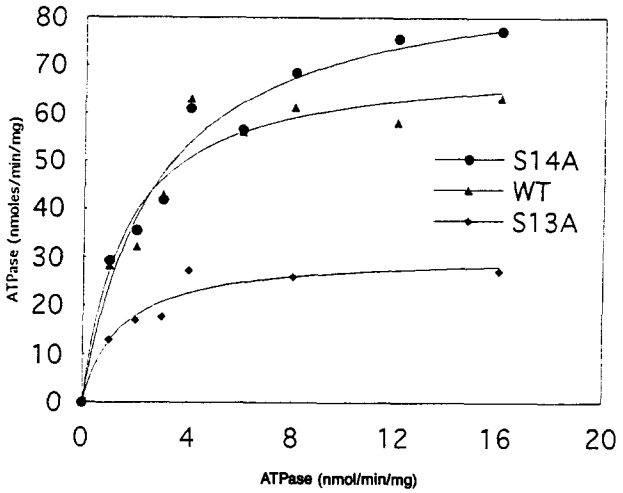


Figure 5. Myosin containing S13A RMLC has reduced actin-activated MgATPase. Myosin purified from cells expressing wild type (*triangle*), S13A (*diamond*), and S14A (*circle*) RMLC were assayed for ATPase activity in the presence of 0–16 μM actin.

plaques did not contain fruiting bodies. Cells grown from non-developing plaques were G418 sensitive indicating they had lost the expression plasmid in the absence of drug selection and reverted to the RMLC null phenotype. When transformants were plated on lawns of a G418 resistant *E. coli* (Hughes et al., 1992) on SM agar containing G418 (100 μg/ml) to maintain selection throughout bacterial growth, normal fruiting bodies appeared in all plaques and contained G418-resistant spores.

S13A Cells Exhibit Normal Myosin Localization

Cells were processed for indirect immunofluorescence and stained with an anti-MHC monoclonal antibody (396). In RMLC null cells, myosin localization is affected in both substrate and suspension cultured cells (Chen et al., 1994). In suspension cultured RMLC null cells myosin is observed to have a primarily endosomal localization in contrast to the primarily cortical localization seen in wildtype cells. When RMLC null cells were grown on a solid substrate myosin was seen in a normal cortical localization or in intense spots generally found near the posterior cortex. In contrast, myosin in the S13A and S14A mutant cells showed a pattern of localization similar to that seen in wild-type transformed cells (Fig. 7). Thus, expression of the S13A and S14A mutant light chains corrected the defect in myosin localization observed in the RMLC null cells (Chen et al., 1994).

S13A RMLC Corrects the Cytokinesis Defect of RMLC Null Cells

When RMLC null cells are grown in suspension they become large and multinucleate due to a defect in cytokinesis (Fig. 8 *A*). Null cells transformed with wild type, S13A, and

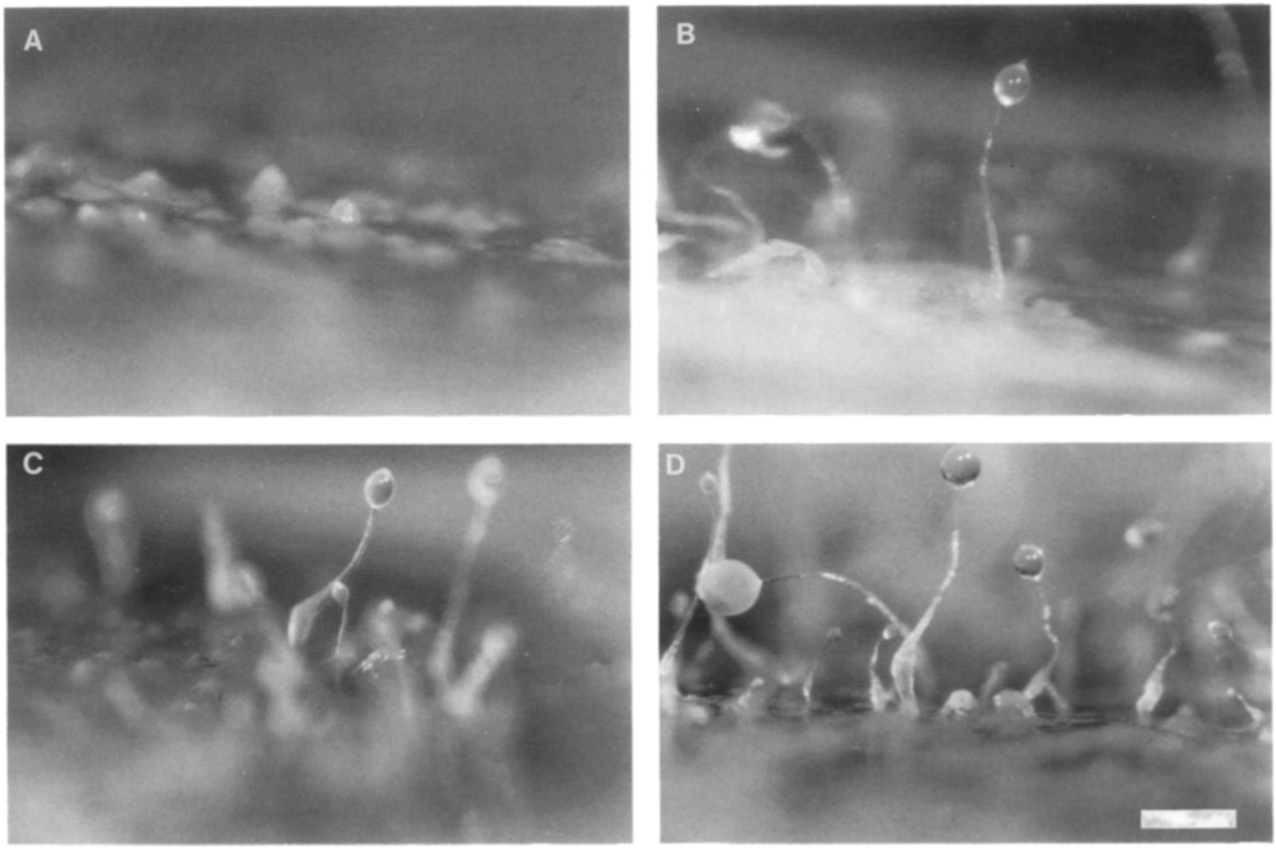


Figure 6. RMLC null cells transformed with S13A and S14A RMLC develop normally. Cells were plated on *K. aerogenes* and allowed to develop. (*A*) RMLC null cells. (*B*) RMLC null cells transformed with wild-type RMLC. (*C*) RMLC null cells transformed with S13A RMLC. (*D*) RMLC null cells transformed with S14A RMLC. Only null cells do not complete development. Bar, 1 mm.

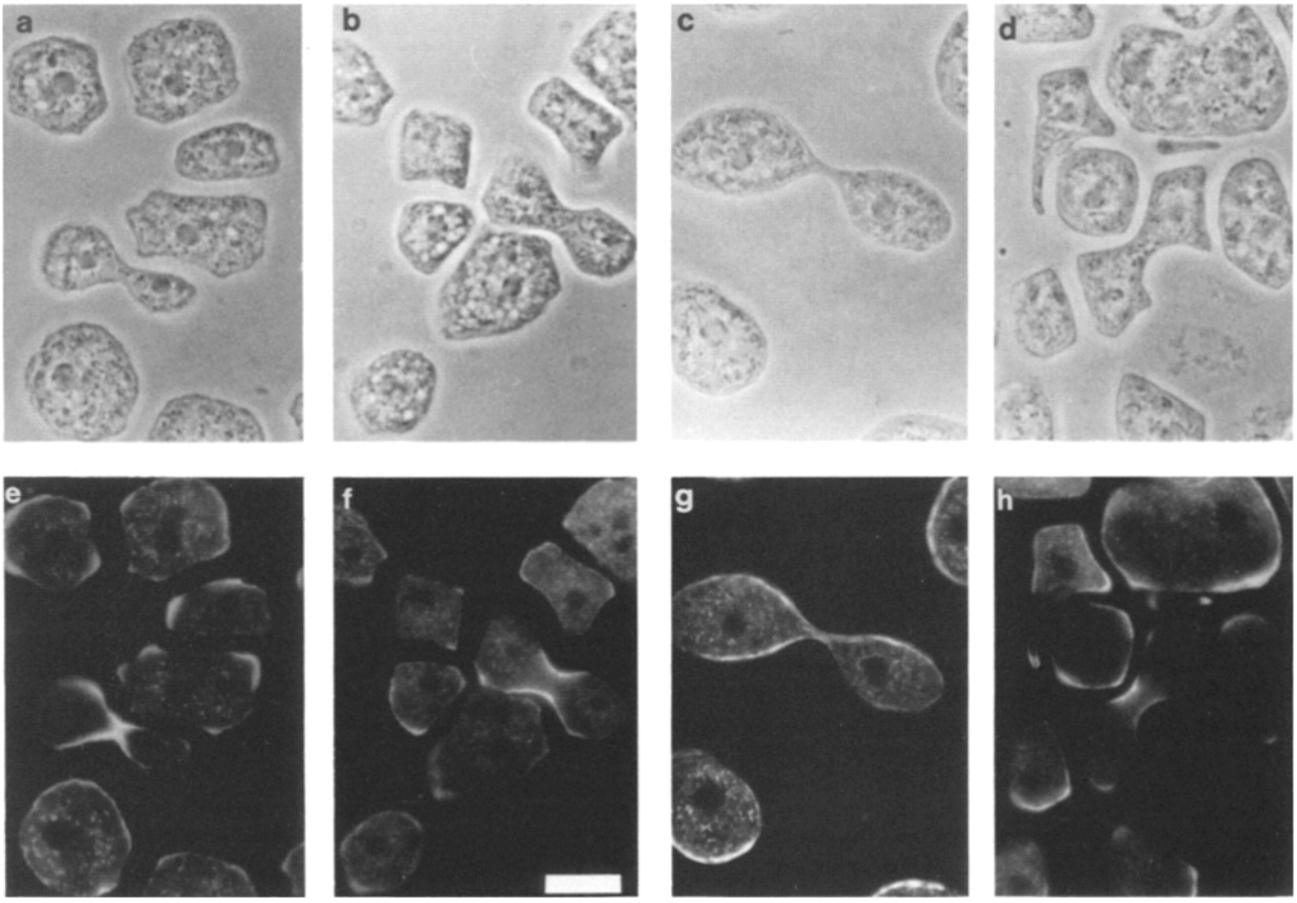


Figure 7. Distribution of myosin in control and mutant cells. Paired phase (*a–d*) and anti-MHC indirect immunofluorescence (*e–h*) photomicrographs. Cells of wild type (*a* and *e*), RMLC null E12 transformed with wild-type RMLC (*b* and *f*), RMLC null E2 transformed with S13A RMLC (*c* and *g*), and RMLC null E12 transformed with S14A RMLC (*d* and *h*). Immunofluorescence of all cells exhibited myosin localization in cortical regions and the contractile ring of dividing cells. RMLC null E12 transformed with S13A RMLC exhibit more cytoplasmic myosin localization than seen in the other cell types. Bar, 10 μm.

S14A remain mostly mononucleate in suspension (Fig. 8, *B*, *C*, and *D*). Null cells transformed with wild type, S13A, and S14A grow with a doubling time of 18 h (Fig. 9). There was a reduction in growth rate of 86% compared to parental cells of strain JH10 which may be due to the presence of the G418 selection drug. Alternatively, the added metabolic burden of high levels of ectopic expression of RMLC could contribute to decreased growth rate.

Discussion

We have identified the site of phosphorylation on the RMLC of *Dictyostelium* myosin and used site directed mutagenesis to replace the phosphorylated serine with a non-phosphorylatable alanine. When this mutant light chain is expressed in an RMLC null cell line created by targeted disruption of the RMLC gene, the mutant RMLC corrects the phenotypic defects of the mlcR$^-$ cells as well as does expression of wild-type RMLC. By immunoprecipitation of myosin from ^{32}P-labeled cells, we were unable to detect phosphorylation of the mutant RMLC. Although the mutant myosin appears capable of performing the normal cellular functions of myosin, it has a significantly reduced actin-activated ATPase, comparable to that observed in wild-type myosin dephosphorylated by phosphatase treatment. These results suggest that the phosphorylation of the RMLC is not required for cellular functions including cytokinesis and cell movements required for multicellular morphogenesis.

Several lines of evidence have suggested the importance of RMLC phosphorylation. First, phosphorylation of the RMLC in smooth muscle and vertebrate nonmuscle systems is required for significant levels of actin-activated ATPase activity (Kamm and Stull, 1985; Trybus, 1989; Tan et al., 1992). Second, RMLC phosphorylation has been shown to regulate the actin-activated ATPase activity of myosins from a variety of sources, including *Dictyostelium*. Phosphorylation also has been shown to regulate motor function as assayed by the movement of actin filaments in vitro (Griffith et al., 1987; Uyeda and Spudich, 1993; Ruppel et al., 1994). Third, the phosphorylation of *Dictyostelium* RMLC is tightly regulated during the aggregation stage of development. Berlot et al. (1985) have shown that phosphorylation of the RMLC increases within 20 s following binding of cyclic AMP to the cell surface cAMP receptor responsible for mediating chemotaxis. The level of RMLC phosphorylation peaks soon thereafter and declines to prestimulation levels by 90–120 s. During this period, 30 s to 2 min following chemotactic stimulation, *Dictyostelium* cells exhibit the so-

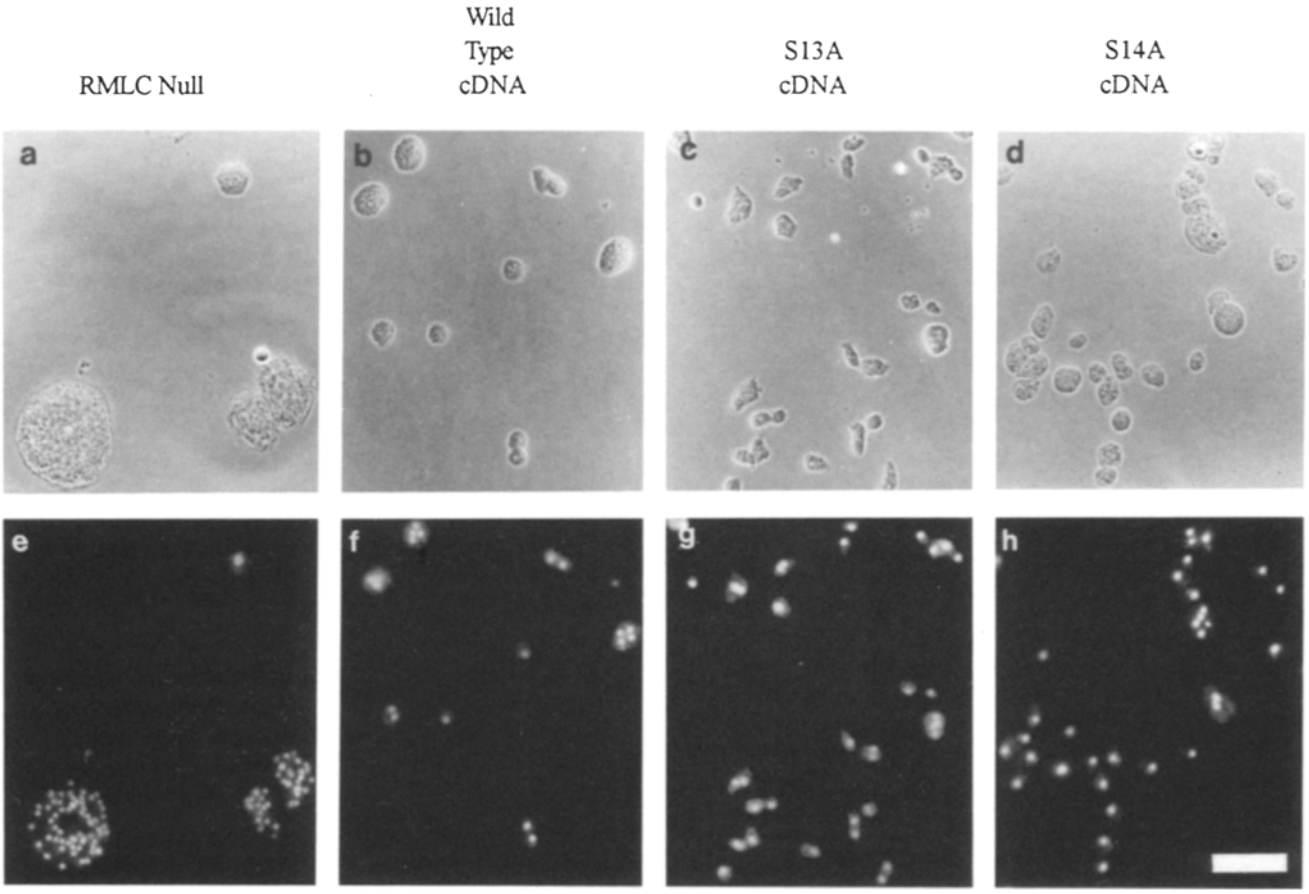


Figure 8. RMLC null cells transformed with S13A RMLC remain mostly mononucleate in suspension. Cells were grown in suspension for 3 d and then fixed, stained with DAPI, and scored for number of nuclei per cell. (*A*) Paired phase (*a–d*) and DAPI fluorescence (*e–h*) photomicrographs. Cells of RMLC null cell E12 (*a* and *e*), RMLC null E12 transformed with wild-type RMLC (*b* and *f*), RMLC null E12 transformed with S13A RMLC (*c* and *g*), and RMLC null E12 transformed with S14A RMLC (*d* and *h*). (*B*) Histogram showing the distribution of the number of nuclei per cell. At least 200 cells of each cell type on two separate coverslips were scored. The average percentage of cells in each category is presented. A majority of null cells have greater than six nuclei per cell, whereas the other cell lines were mostly mono- or binucleate. ■, RMLC null; □, wild-type cDNA; ▨, S13A cDNA; ▨, S14A cDNA. Bar, 30 μm.

called cringe response, in which they round up and stop moving. Fukui and colleagues (Yumura and Fukui, 1985; Nachmias et al., 1989) have also shown that the pattern of myosin staining changes from a predominantly endoplasmic localization to a primarily cortical one in response to chemoattractant. Finally, *Dictyostelium* expresses a myosin light chain kinase which is highly specific for the *Dictyostelium* RMLC (Griffith ėt al., 1987). Taken together, these observations are consistent with a role for RMLC phosphorylation in development.

Berlot et al. (1987) demonstrated that the RMLC is phosphorylated on serine. Based on sequence homology with other RMLCs, it was previously suggested that S14 was the probable phosphorylation site (Tafuri et al., 1989; see also Tan et al., 1992, Fig. 3). Based on considerations of the local sequence environment surrounding the phosphorylation site, Kobayashi et al. (1991) proposed that the *Dictyostelium* RMLC was phosphorylated at S13. By comparing amino acid sequence of *E. coli*–expressed RMLC before and after treatment with cloned *Dictyostelium* MLCK, we have determined that S13 is phosphorylated by cloned MLCK in vitro. The identification of the MLCK phosphorylation site was confirmed by our observation that a recombinant RMLC, carrying a serine 13 to alanine mutation, expressed in *E. coli*, was no longer phosphorylated to any significant degree following treatment with purified *Dictyostelium* MLCK.

Several studies have shown that the level of phosphorylation regulates the actin-activated ATPase and the ability of *Dictyostelium* myosin to produce ATP dependent movement of actin filaments in vitro (Griffith et al., 1987; Uyeda and

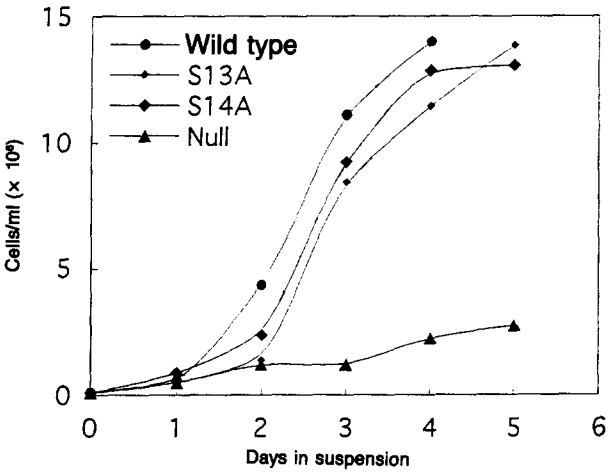


Figure 9. RMLC null cells transformed with S13A RMLC grow in suspension with normal doubling times. Cells were inoculated in flasks and shaken for 5 d. Aliquots were counted on each day and plotted as 10^6 cells/ml. RMLC null cells double only twice in 5 d, whereas the other cell lines exhibit exponential growth and saturation.

Spudich, 1993; Ruppel et al., 1994). When purified from vegetative *Dictyostelium* cells, the RMLC typically carries about 0.3 mol phosphate per mole of RMLC polypeptide. In contrast, myosin purified from mlcR$^-$ cells expressing S13A RMLC showed no light chain phosphorylation. Both immunoprecipitation of myosin from ^{32}P-labeled cells and urea-glycerol gel analysis of purified myosin (data not shown) showed no evidence of phosphorylation on S13A RMLC. Myosin carrying the S13A RMLC exhibited levels of calcium ATPase comparable to wild type, although the level of actin-activated ATPase activity was only 25–30% that of wild-type type. Phosphatase treated myosin carrying less than 0.01 mol phosphate per mole of RMLC showed ATPase activities of ~20 nmol/min/mg protein (Griffith et al., 1987), corresponding to a turnover rate of ~0.09 per s. S13A myosin purified from vegetative cells had comparable levels of actin-activated ATPase activity (also 0.09 per s). This level of actin-activated ATPase activity is also in the range obtained by steady state measurements of dephosphorylated smooth muscle myosin (Sellers et al., 1981; Trybus, 1989). Thus it appears that the S13A RMLC mutation produced the anticipated result: myosin with actin-activated ATPase activity comparable to unphosphorylated myosin. However, despite the reduced actin-activated ATPase, the S13A RMLC myosin had sufficient function in vivo to rescue the phenotypic defects of the RMLC null cells. RMLC null cells expressing the S13A mutant myosin grew in suspension with a doubling time comparable to cells rescued by wild-type RMLC, and progressed normally through the aggregation and multicellular stages of *Dictyostelium* development.

It has been proposed that the RMLC can be phosphorylated by kinases other than the MLCK, including both PKC and p34^{cdc2} kinase. The sequence of the RMLC suggests there are potential sites for phosphorylation by PKC at Ser-3 or Thr-4 and by p34^{cdc2} kinase at Ser-3. Since the S13A mutant shows no detectable phosphorylation, it seems unlikely that a significant portion of the RMLC in growing cells is phosphorylated at one or more of these sites. However, a cell cycle-dependent phosphorylation such as has been suggested by Satterwhite et al. (1992) may not have been detectable in our experiments. Assuming the cell population is randomly distributed throughout the cell cycle (which is a minimum of 8 h long) and the period of mitosis where the RMLC would be phosphorylated is shorter than 10–15 min, the phosphorylation would be below our limit of detection. There has been concern that the characterized *Dictyostelium* MLCK may not be the only kinase capable of phosphorylating the RMLC (Silveira, L. A., J. A. Smith, and J. A. Spudich, personal communication). Our inability to detect phosphorylation on the S13A mutant suggests that regardless of the kinase responsible for phosphorylation, S13 must be the predominant phosphorylation site on *Dictyostelium* RMLC.

There have been many studies demonstrating the importance of RMLC phosphorylation for the biochemical properties of myosin thought to correlate with in vivo contractile activity. Many of these studies have used desensitized scallop myosin hybrids reconstituted with foreign light chains (Kendrick-Jones et al., 1976; Sellers et al., 1980; Reinach et al., 1986), or myosin from which RMLC was removed or exchanged (Lowey et al., 1993; Trybus and Chatman, 1993; Trybus et al., 1994). These experiments and others have shown that phosphorylation increases the actin activated ATPase of smooth muscle and non-muscle myosins, including *Dictyostelium* myosin. In addition, phosphorylation is coupled to the initiation of contraction in smooth muscle (Hartshorne and Siemankowski, 1981) and has been shown to promote a conformational change that converts folded myosin monomers into an extended form that is assembly competent.

Given this wealth of in vitro data it seems surprising that a phosphorylation site deficient RMLC functions sufficiently well to complement all of the tested phenotypic defects of a RMLC null mutant. These results question the significance of RMLC phosphorylation in vivo. While it is possible that *Dictyostelium* is unusual with regard to the function of RMLC phosphorylation, it is comparable to other myosins in structure (Warrick et al., 1986; Pasternak et al., 1989; Schroder et al., 1993), and in the levels of ATPase activity and motor activity as indicated by rates of movement it produces in in vitro motility assays (Griffith et al., 1987; Uyeda and Spudich, 1993; Ruppel et al., 1994). *Dictyostelium* myosin may be unusual in that filament assembly is regulated by myosin heavy chain phosphorylation (Kuczmarski and Spudich, 1980; Egelhoff et al., 1993), and that the myosin light chain kinase is calcium independent (Griffith et al., 1987; Tan and Spudich, 1990). In addition, the magnitude of phosphorylation dependent enhancement of actin activated ATPase activity may be somewhat lower for *Dictyostelium* myosin than for smooth muscle myosin. However, these measurements have been of steady state activity, and may result in an underestimation of the phosphorylation-dependent activation of *Dictyostelium* myosin as has been shown for smooth muscle myosin steady-state measurements (Sellers, 1985). Alternatively these results may suggest that RMLC phosphorylation plays a more subtle role in regulation than would be detected by the relatively crude assays of growth rate and development timing and competence. For example, phosphorylation could make cytokinesis more efficient by 5%, producing a 5% increase in growth rate, which would be difficult to document, but which might lead

to a relatively strong evolutionary selection pressure for retaining this regulatory process. In this light it is interesting to note the recent report by Uyeda and Spudich (1993) that a mutant *Dictyostelium* myosin heavy chain lacking the RMLC-binding domain complements most of the phenotypic defects of a myosin heavy chain null mutant. This mutant myosin has somewhat increased ATPase activity and produced normal rates of actin filament movement using an in vitro motility assay. These results provide independent support for the hypothesis that the regulatory role of RMLC phosphorylation in *Dictyostelium* is not essential. However, the Uyeda and Spudich results taken together with our report that an mlcR$^-$ cell exhibits many of the defects of mhcA$^-$ cells (Chen et al., 1994), make it likely that some function provided by the RMLC, perhaps a subtle modulation of function, provides sufficient selective advantage to assure the maintenance of the RMLC and myosin light chain kinase through evolution.

We would like to thank R. Pollenz for the mutated actin 15 promoter; K. Flynn for producing the 1A2 antibody; Dr. G. Gerisch for the 396 antibody; Drs. J. L. Tan, J. Smith, and J. A. Spudich, Stanford University for the MLCK; Dr. J. Smith for sharing her protocol for in vivo labeling of *Dictyostelium*; Dr. K.-L. Ngai and J. Burrows of the Northwestern University Biotech Facility for performing the amino acid sequencing and providing oligonucleotides; Dr. T.-L. Chen for providing the rabbit actin and critical reading of the manuscript; Dr. C. Collins for suggesting the ATPase assay modifications; Dr. J. Bartles for access to the densitometer; and M. Chaudoir for help with the plasmid map.

This work was sponsored by National Institutes of Health grant GM39264.

Received for publication 13 April 1994 and in revised form 26 September 1994.

References

Berlot, C. H., J. A. Spudich, and P. N. Devreotes. 1985. Chemoattractant-elicited increases in myosin phosphorylation in *Dictyostelium*. *Cell*. 43:307–314.

Berlot, C. H., P. N. Devreotes, and J. A. Spudich. 1987. Chemoattractant-elicited increases in *Dictyostelium* myosin phosphorylation are due to changes in myosin localisation and increases in kinase activity. *J. Biol. Chem.* 262:3918–3926.

Chen, P., B. D. Ostrow, S. R. Tafuri, and R. L. Chisholm. 1994. Targeted disruption of the *Dictyostelium* RMLC gene produces cells defective in cytokinesis and development. *J. Cell Biol.* submitted.

Clarke, M., and J. A. Spudich. 1974. Biochemical and structural studies of actomyosin-like proteins from non-muscle cells: isolation and characterization of myosin from amoebae of *Dictyostelium discoideum*. *J. Mol. Biol.* 86:209–222.

Claviez, M., K. Pagh, H. Maruta, W. Baltes, P. Fisher, and G. Gerisch. 1982. Electron microscopic mapping of monoclonal antibodies on the tail region of *Dictyostelium* myosin. *EMBO (Eur. Mol. Biol. Organ.) J.* 1:1017–1022.

Côté, G. P., and U. Bukiejko. 1987. Purification and characterization of a myosin heavy chain kinase from *Dictyostelium discoideum*. *J. Biol. Chem.* 262:1065–1072.

DeLozanne, A., and J. A. Spudich. 1987. Disruption of the *Dictyostelium discoideum* myosin heavy chain gene by homologous recombination. *Science (Wash. DC)*. 236:1086–1091.

Egelhoff, T. T., R. J. Lee, and J. A. Spudich. 1993. Mutagenesis of *Dictyostelium* myosin heavy chain phosphorylation sites: direct evidence that these sites regulate myosin filament assembly and localisation in vivo. *Cell*. 75:363–371.

Egelhoff, T. T., S. S. Brown, and J. A. Spudich. 1991. Spatial and temporal control of nonmuscle myosin localization: identification of a domain that is necessary for myosin filament disassembly in vivo. *J. Cell Biol.* 112: 677–688.

Evan, G. I., G. K. Lewis, G. Ramsay, and J. M. Bishop. 1985. Isolation of monoclonal antibodies specific for human c-*myc* proto-oncogene product. *Mol. Cell. Biol.* 5:3610–3616.

Fukui, Y. 1990. Actomyosin organization in mitotic *Dictyostelium* amebae. *Ann. NY Acad. Sci.* 582:156–165.

Fukui, Y., and S. Yumura. 1986. Actomyosin dynamics in chemotactic amebae boid movement in *Dictyostelium*. *Cell Motil. Cytoskeleton*. 6:662–673.

Fukui, Y., A. DeLozanne, and J. A. Spudich. 1990. Structure and function of the cytoskeleton of a *Dictyostelium* myosin-defective mutant. *J. Cell Biol.* 110:367–378.

Futrelle, R. P., J. Traut, and W. G. McKee. 1982. Cell behavior in *Dictyostelium discoideum*: preaggregation response to localized cyclic AMP pulses. *J. Cell Biol.* 92:807–821.

Griffith, L. M., S. M. Downs, and J. A. Spudich. 1987. Myosin light chain kinase and myosin light chain phosphatase from *Dictyostelium*: effects of reversible phosphorylation on myosin structure and function. *J. Cell Biol.* 104:1309–1323.

Hartshorne, D. J. and R. F. Siemankowski. 1981. Regulation of smooth muscle actomyosin. *Annu. Rev. Physiol.* 43:519–530.

Howard, P. K., K. G. Ahern, and R. A. Firtel. 1988. Establishment of a transient expression system for *Dictyostelium discoideum*. *Nucleic Acids Res.* 16:2613–2623.

Hughes, J. E., G. J. Podgorski, and D. L. Welker. 1992. Selection of *Dictyostelium discoideum* transformants and analysis of vector maintenance using live bacteria resistant of G418. *Plasmid*. 28:46–60.

Ikebe, M., D. J. Hartshorne, and M. Elzinga. 1987. Phosphorylation of the 20,000-dalton light chain of smooth muscle myosin by the calcium-activated, phospholipid-dependent protein kinase: phosphorylation sites and effects of phosphorylation. *J. Biol. Chem.* 262:9569–9573.

Kamm, K. E., and J. T. Stull. 1985. The function of myosin and myosin light chain kinase phosphorylation in smooth muscle. *Annu. Rev. Pharmacol. Toxicol.* 25:593–620.

Karess, R. E., X.-J. Chang, K. A. Edwards, S. Kulkarni, I. Aguilera, and D. P. Kiehart. 1991. The regulatory light chain of nonmuscle myosin is encoded by *spaghetti-squash*, a gene required for cytokinesis in *Drosophila*. *Cell*. 65:1177–1189.

Kendric-Jones, J., E. M. Szentkiralyi, and A. G. Szent-Gyorgyi. 1976. Regulatory light chains in myosins. *J. Mol. Biol.* 104:747–775.

Kiehart, D., I. Mabuchi, and S. Inoue. 1982. Evidence that myosin does not contribute to force production in chromosome movement. *J. Cell Biol.* 94:165–178.

King, S. M., T. Otter, and G. B. Witman. 1986. Purification and characterization of *Chlamydomonas* flagellar dyneins. *Methods Enzymol.* 134:291–306.

Knecht, D. A., and W. F. Loomis. 1987. Antisense RNA inactivation of myosin heavy chain gene expression in *Dictyostelium discoideum*. *Science (Wash. DC)*. 236:1081–1086.

Knecht, D. A., S. M. Cohen, W. F. Loomis, and H. F. Lodish. 1986. Developmental regulation of *Dictyostelium discoideum* actin gene fusions carried on low-copy and high copy transformation vectors. *Mol. Cell Biol.* 6: 3973–3983.

Kobayashi, T., H. G. Zot, T. D. Pollard, and J. H. Collins. 1991. Functional implications of the unusual amino acid sequence of the regulatory light chain of *Acanthamoeba castellanii* myosin-II. *J. Mus. Res. Cell Motil.* 12: 553–559.

Korn, E. D., and J. A. Hammer. 1988. Myosins of nonmuscle cells. *Ann. Rev. Biophys. Biophys. Chem.* 17:23–45.

Kuczmarski, E. R., and J. A. Spudich. 1980. Regulation of myosin self-assembly: phosphorylation of *Dictyostelium* heavy chains inhibits formation of thick filaments. *Proc. Nat. Acad. Sci. USA*. 77:7292–7296.

Kuczmarski, E. R., S. R. Tafuri, and L. M. Parysek. 1987. Effects of heavy chain phosphorylation on the polymerization and structure of *Dictyostelium* myosin filaments. *J. Cell Biol.* 105:2989–2997.

Kuczmarski, E. R., and J. Pagone. 1986. Myosin specific phosphatases isolated from *Dictyostelium discoideum*. *J. Muscle Res. Cell Motil.* 7:510–516.

Lowey, S., G. S. Waller, and K. M. Trybus. 1993. Skeletal muscle myosin light chains are essential for physiological speeds of shortening. *Nature (Lond.)*. 365:454–456.

Luck-Vielmetter, D., M. Schleicher, B. Grabatin, J. Wippler, and G. Gerisch. 1990. Replacement of threoning residues by serine and alanine in a phosphorylatable heavy chain fragment of *Dictyostelium* myosin II. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 269:239–243.

Mabuchi, I., and M. Okuno. 1977. The effect of myosin antibody on the division of starfish blastomeres. *J. Cell Biol.* 74:251–263.

Manstein, D. J., M. A. Titus, A. DeLozanne, and J. A. Spudich. 1989. Gene replacement in *Dictyostelium*: generation of myosin null mutants. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:923–932.

McKeown, M., and R. A. Firtel. 1981. Evidence for sub-families of actin genes in *Dictyostelium* as determined by comparisons of 3′ end sequences. *J. Mol. Biol.* 151:593–606.

Nachmias, V. T., Y. Fukui, and J. A. Spudich. 1989. Chemoattractant-elicited translocation of myosin in motile *Dictyostelium*. *Cell Motil. Cytoskeleton*. 13:158–169.

Pagh, K., and G. Gerisch. 1986. Monoclonal antibodies binding to the tail of *Dictyostelium discoideum* myosin: their effects on antiparallel and parallel assembly and actin-activated ATPase activity. *J. Cell Biol.* 103:1527–1538.

Pardee, J. D., and J. A. Spudich. 1982. Purification of muscle actin. *Methods Enzymol.* 85:164–181.

Pasternak, C., P. F. Flicker, S. Ravid, and J. A. Spudich. 1989. Intermolecular versus intramolecular interactions of *Dictyostelium* myosin: possible regulation by heavy chain phosphorylation. *J. Cell Biol.* 109:203–210.

Perrie, W. T., and S. V. Perry. 1970. An electrophoretic study of the low-

molecular-weight components of myosin. *Biochem. J.* 119:31–38.
Pollard, T. D., and E. D. Korn. 1973. *Acanthameba* myosin: I. Isolation from *Acanthameba castellanii* of an enzyme similar to muscle myosin. *J. Biol. Chem.* 248:4682–4690.
Pollenz, R. S., T.-L. L. Chen, L. Trivinos-Lagos, and R. L. Chisholm. 1992. The *Dictyostelium* essential light chain is required for myosin function. *Cell.* 69:951–962.
Rayment, I., W. R. Rypniewshi, K. Schmidt-Base, R. Smith, D. R. Tomchick, M. M. Benning, D. A. Winkelmann, G. Wesenberg, and H. H. Holden. 1993. Three-dimensional structure of myosin subfragment-1: a molecular motor. *Science (Wash. DC).* 261:50–58.
Reinach, F. C., K. Nagai, and J. Kendrick-Jones. 1986. Site-directed mutagenesis of the regulatory light-chain Ca^{2+}/Mg^{2+} binding site and its role in hybrid myosins. *Nature (Lond.).* 322:80–83.
Roach, P. J., and Y. Wang. 1991. Identification of phosphorylation sites in peptides suing a microsequencer. *Methods Enzymol.* 201:200–206.
Ruppel, K. M., T. Uyeda, and J. Spudich. 1994. Role of highly conserved lysine 130 of myosin motor domain. In vivo and in vitro characterization of site specifically mutated myosin. *J. Biol. Chem.* 269:18773–18780.
Satterwhite, L. L., M. J. Lohka, K. L. Wilson, T. Y. Scherson, L. J. Cisek, J. L. Corden, and T. D. Pollard. 1992. Phosphorylation of myosin-II regulatory light chain by cyclin-$p34^{cdc2}$: a mechanism for the timing of cytokinesis. *J. Cell Biol.* 118:595–605.
Schroder, R. R., D. J. Manstein, W. Jahn, H. Holden, I. Rayment, K. C. Holmes, and J. A. Spudich. 1993. Three-dimensional atomic model of F-actin decorated with *Dictyostelium* myosin S1. *Nature (Lond.).* 364: 171–174.
Sellers, J. R., M. D. Pato, and R. S. Adelstein. 1981. Reversible phosphorylation of smooth muscle myosin, heavy meromyosin, and platelet myosin. *J. Biol. Chem.* 256:13137–13142.
Sellers, J. R., P. D. Chantler, and A. G. Szent-Gyorgyi. 1980. Hybrid formation between scallop myofibrils and foreign regulatory light chains. *J. Mol. Biol.* 144:223–245.
Sellers, J. R. 1985. Mechanism of the phosphorylation-dependent regulation of smooth muscle heavy meromyosin. *J. Biol. Chem.* 260:15815–15819.
Somlyo, A. P., and A. V. Somlyo. 1981. Effects and subcellular distribution of magnesium in smooth and striated muscle. *Fed. Proc.* 40:2667–2671.
Stappenbeck, T. S., and K. J. Green. 1992. The desmoplakin carboxyl terminus coaligns with and specifically disrupts intermediate filament networks when expressed in cultured cells. *J. Cell Biol.* 116:1197–1209.
Stendahl, O., J. H. Hartwig, E. A. Brotschi, and T. P. Stossel. 1980. Distribution of actin-binding protein and myosin in macrophages during spreading and phagocytosis. *J. Cell Biol.* 84:215–224.
Suzuki, H., H. Onishi, K. Takahashi, and S. Watanabe. 1978. Structure and function of chicken gizzard myosin. *J. Biochem.* 84:1529–1542.
Tafuri, S. R., A. M. Rushforth, E. R. Kuczmarski, and R. L. Chisholm. 1989. *Dictyostelium discoideum* myosin: isolation and characterization of cDNAs encoding the regulatory light chain. *Mol. Cell. Biol.* 9:3073–3080.
Tan, J., and J. Spudich. 1990. *Dictyostelium* myosin light chain kinase. *J. Biol. Chem.* 265:13818–13824.
Tan, J., and J. A. Spudich. 1991. Characterization and bacterial expression of the *Dictyostelium* myosin light chain cDNA. Identification of an autoinhibitory domain. *J. Biol. Chem.* 266:16044–16049.
Tan, J. L., S. Ravid, and J. A. Spudich. 1992. Control of nonmuscle myosins by phosphorylation. *Annu. Rev. Biochem.* 61:721–759.
Truong, T., Q. G. Medley, and G. P. Côté. 1992. Actin-activated Mg-ATPase activity of *Dictyostelium* myosin II. *J. Biol. Chem.* 267:9767–9772.
Trybus, K. M., and S. Lowey. 1987. Assembly of smooth muscle myosin minifilaments: effects of phosphorylation and nucleotide binding. *J. Cell Biol.* 105:3007–3019.
Trybus, K. M. 1989. Filamentous smooth muscle myosin is regulated by phosphorylation. *J. Cell Biol.* 109:2887–2894.
Trybus, K. M., and T. A. Chatman. 1993. Chimeric regulatory light chains as probes of smooth muscle myosin function. *J. Biol. Chem.* 268:4412–4419.
Trybus, K., G. Waller, and T. Chatman. 1994. Coupling of ATPase activity and motility in smooth muscle myosin is mediated by the regulatory light chain. *J. Cell Biol.* 124:963–969.
Uyeda, T. Q. P., and J. A. Spudich. 1993. A functional recombinant myosin II lacking a regulatory light chain-binding site. *Science (Wash. DC).* 262:1867–1870.
Uyemura, D. G., S. S. Brown, and J. A. Spudich. 1978. Biochemical and structural characterisation of actin from *Dictyostelium discoideum*. *J. Biol. Chem.* 253:9088–9096.
Vaillancourt, J. P., C. Lyons, and C. P. Côté. 1988. Identification of two phosphorylated threonines in the tail region of *Dictyostelium* myosin II. *J. Biol. Chem.* 263:10082–10087.
Varnum-Finney, B. J., E. Voss, and D. R. Soll. 1987. Frequency and orientation of pseudopod formation of *Dictyostelium discoideum* amebae chemotaxing in a spatial gradient: further evidence for a temporal mechanism. *Cell Motil. Cyoskeleton.* 8:18–26.
Vibert, P., and C. Cohen. 1988. Domains, motions, and regulation in the myosin head. *J. Mus. Res. Cell Motil.* 9:296–305.
Warrick, H. M., and J. A. Spudich. 1987. Myosin structure and function in cell motility. *Annu. Rev. Cell Biol.* 3:379–421.
Warrick, H. M., A. DeLozanne, L. A. Leinwand, and J. A. Spudich. 1986. Conserved protein domains in a myosin heavy chain gene from *Dictyostelium discoideum*. *Proc. Natl. Acad. Sci. USA.* 83:9433–9437.
Wessels, D., D. R. Soll, D. Knecht, W. F. Loomis, A. DeLozanne, and J. A. Spudich. 1988. Cell motility and chemotaxis in *Dictyostelium* amebae lacking myosin heavy chain. *Dev. Biol.* 128:164–177.
Yumura, S., and Y. Fukui. 1985. Reversible cyclic AMP-dependent change in distribution of myosin thick filaments in *Dictyostelium*. *Nature (Lond.).* 314:194–196.