

Targeted Disruption of the *Dictyostelium* RMLC Gene Produces Cells Defective in Cytokinesis and Development

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Abstract. Conventional myosin has two different light chains bound to the neck region of the molecule. It has been suggested that the light chains contribute to myosin function by providing structural support to the neck region, therefore amplifying the conformational changes in the head following ATP hydrolysis (Rayment et al., 1993). The regulatory light chain is also believed to be important in regulating the actin-activated ATPase and myosin motor function as assayed by an in vitro motility assay (Griffith et al., 1987). Despite extensive in vitro biochemical study, little is known regarding RMLC function and its regulatory role in vivo. To better understand the importance and contribution of RMLC in vivo, we engineered *Dictyostelium* cell lines with a disrupted RMLC gene. Homologous recombination between the introduced gene disruption vector and the chromosomal RMLC locus (*mlcR*) resulted in disruption of the RMLC-coding region, leading to cells devoid of both the RMLC transcript and the 18-kD RMLC poly-

peptide. RMLC-deficient cells failed to divide in suspension, becoming large and multinucleate, and could not complete development following starvation. These results, similar to those from myosin heavy chain mutants (DeLozanne et al., 1987; Manstein et al., 1989), suggest the RMLC subunit is required for normal cytokinesis and cell motility. In contrast to the myosin heavy chain mutants, however, the *mlcR* cells are able to cap cell surface receptors following concanavalin A treatment. By immunofluorescence microscopy, RMLC null cells exhibited myosin localization patterns different from that of wild-type cells. The myosin localization in RMLC null cells also varied depending upon whether the cells were cultured in suspension or on a solid substrate. In vitro, purified RMLC⁻ myosin assembled to form thick filaments comparable to wild-type myosin, but the filaments then exhibited abnormal disassembly properties. These results indicate that in vivo RMLC is necessary for myosin function.

MYOSIN, a principal protein component of contractile systems (Adelstein et al., 1980; Harrington and Rogers, 1984), is widely distributed in eukaryotic cells. It has been implicated in critical motile processes including cell locomotion, cytoplasmic streaming, cytokineses, and many other functions (Loomis, 1977; Taylor and Condeelis, 1979; Yumura and Fukui, 1985; Fukui and Yumura, 1986; DeLozanne and Spudich, 1987; Fukui et al., 1990). The myosin molecule consists of six subunits: two myosin heavy chains (MHC),¹ two regulatory myosin light chains (RMLC), and two essential myosin light chains

(EMLC). One RMLC and one EMLC are non-covalently associated with the neck region of each myosin head (Winkelman and Lowey, 1986; Katoh and Lowey, 1989). A 2.8-Å resolution structure of the chicken skeletal muscle myosin head locates both light chains to an α -helix which emerges from the C-terminus of the globular head domain. The EMLC is placed more proximal to the nucleotide-binding site, while the RMLC is located more distally (Rayment et al., 1993).

Two classes of RMLC can be distinguished based upon their regulatory function (Rowe and Kendrick-Jones, 1992). Skeletal muscle contraction is controlled primarily by actin-linked regulation in which Ca²⁺ binds to the thin filament-associated troponin-tropomyosin complex. However, phosphorylation of skeletal muscle RMLC has been shown to increase the rate at which the myosin enters the force production state (Sweeney et al., 1993). In contrast, smooth muscle and non-muscle systems are regulated by a myosin-linked system in which phosphorylation of RMLC increases actin-activated myosin ATPase activity (Griffith et al., 1987; Trybus, 1991). At physiological ionic strength and pH, smooth

In this paper, the word "myosin" represents myosin II only, unless otherwise noted.

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1. *Abbreviations used in this paper:* EMLC, essential myosin light chain; MHC, myosin heavy chain; *mlcR*⁻, RMLC null mutant; RMLC, regulatory myosin light chain; RMLC⁻ myosin, myosin without the regulatory light chain.

muscle myosin with unphosphorylated RMLC exists in a folded monomer conformation (Onishi and Wakabayashi, 1982; Trybus et al., 1982; Craig et al., 1983). RMLC phosphorylation promotes filament assembly by converting the folded myosin monomers into an extended assembly-competent configuration. Furthermore, this phosphorylation is coupled to the initiation of contraction (Hartshorne and Siemankowski, 1981). Phosphorylation of non-muscle myosin RMLC has been implicated in the regulation of platelet aggregation (Daniel et al., 1981, 1984), and capping in lymphocytes (Bourguignon et al., 1981). In *Dictyostelium discoideum*, myosin filament assembly is regulated by phosphorylation of the heavy chain (Kuczmarski and Spudich, 1980; Kuczmarski et al., 1987; Egelhoff et al., 1993). In addition, cyclic AMP stimulation induces a rapid and transient RMLC phosphorylation (Berlot et al., 1987), which increases myosin ATPase activity, and dramatically enhances the ability of myosin motor activity as assayed by an in vitro motility assay (Griffith et al., 1987). The ability to exchange heterologous light chains into scallop, skeletal, and smooth muscle myosin in vitro has been exploited to demonstrate the importance and contribution of RMLC subdomains (Reinach et al., 1986; Goodwin et al., 1990; Kendrick-Jones et al., 1991; Messer and Kendrick-Jones, 1991; Rowe and Kendrick-Jones, 1992; Saraswat et al., 1992).

Direct proof that the non-muscle myosin RMLC is required for cytokinesis has recently emerged from genetic studies with *Drosophila* (Karess et al., 1991). The mutation *spaghetti-squash* produces an embryonic lethal phenotype, characterized by a high proportion of polyploid cells in rapidly dividing tissues such as brain, imaginal disks, and gonads. Little is known about the biochemical properties of the *spaghetti-squash* myosin, or the consequences of this mutation for other myosin-dependent cellular properties.

The simple eukaryote *Dictyostelium discoideum* displays locomotion and chemotaxis typical of mammalian cells (Devreotes and Zigmond, 1988), and serves as an excellent model for biochemical and cell biological studies of cell locomotion. The success of gene targeting technology in *Dictyostelium* has created a unique opportunity to study components of cell locomotion in vivo. Both the conventional myosin heavy chain (DeLozanne and Spudich, 1987; Knecht and Loomis, 1987; Manstein et al., 1989) and its essential light chain have been eliminated via both homologous recombination (Chen, T.-L. L., G. Ho, P. Kowalczyk, and R. L. Chisholm, manuscript in preparation) and antisense RNA overexpression (Pollenz et al., 1992). While the mutants are still capable of pseudopod extension and retraction, cell migration, and chemotaxis, all three of these behaviors are markedly impaired in the myosin heavy chain mutants (Wessels et al., 1988). The mutants also showed defects in cytokinesis, receptor capping, and development, defining each of these phenomena as being myosin dependent. Recently, Uyeda and Spudich (1993) generated a *Dictyostelium* cell line in which the wild-type myosin II heavy chain is replaced with a mutant form that lacks the RMLC binding site. Cells expressing this mutant heavy chain can carry out cytokinesis in suspension and nearly normal development, despite the absence of RMLC binding to the recombinant heavy chain. This result contrasts to the requirement of RMLC for cytokinesis, as indicated by the *Drosophila spaghetti-squash* mutant. Information regarding the role of

RMLC function in *Dictyostelium* should be valuable in reconciling these results.

The aim of this study was to explore the consequence of eliminating RMLC function in vivo and correlate this with the in vitro activities of RMLC⁻ myosin. We have disrupted the single *Dictyostelium* *mlcR* gene by homologous recombination. The resulting RMLC null cells (*mlcR*⁻) exhibited defects in cytokinesis and development similar to the MHC mutants. In contrast, *mlcR*⁻ cells were able to cap cell surface receptors after concanavalin A (con A) treatment. These *mlcR*⁻ cells showed abnormal myosin localization by immunofluorescence microscopy, and RMLC⁻ myosin displayed abnormal disassembly properties in vitro.

Material and Methods

Cell Culture

Dictyostelium discoideum strain JH10, created by gene disruption of the *thyl* locus (Hadwiger and Firtel, 1992), was cultured in HL-5 media containing 100 µg/ml thymidine. *mhcA*⁻ cells were cultured according to Manstein et al. (1989).

General Molecular Biology

Standard molecular biological techniques were performed using common procedures (Sambrook et al., 1989). Molecular genetic techniques for working with *Dictyostelium* have also been described by Manstein et al. (1989) and Pollenz et al. (1992).

DNA Constructs and Transformation

Two genomic DNA fragments, a 1.7-kb HindIII fragment containing the 3' half of the *mlcR* gene, and a 2.5-kb EcoRI-HindIII fragment containing the 5' portion of the gene, were isolated by cloning size fractionated EcoRI/HindIII and HindIII digests of *Dictyostelium* DNA as previously described (Pollenz and Chisholm, 1991). The cloned genomic DNA is indicated by a thick line in Fig. 1. The *mlcR* gene is contained on fragments which contain ~2.1 kb of 5' flanking and 1 kb of 3' flanking sequence. The coding sequence also contains two introns of ~200 and 320 bp, respectively, one immediately after the ATG translation initiation site and a second between amino acid residues 91 and 92 of the RMLC coding region. The gene replacement vector, *prmlc-thyl*, was generated by first constructing *prmlc5'* which contained a 2.5-kb EcoRI-HindIII *mlcR* 5' genomic fragment. A 1.7-kb *mlcR3'* genomic HindIII fragment was then ligated into the HindIII site to construct vector *prmlc*. Clones containing properly oriented *mlcR3'* fragment were identified and confirmed by PCR and restriction digestion. Plasmid *prmlc* was then linearized at the StuI site, and a 3.0-kb blunt-ended BamHI-HindIII fragment containing the *thyl* gene (Dynes and Firtel, 1989) was introduced by a blunt-end ligation. The resultant vector, *prmlc-thyl*, was then digested with MscI and XhoI. The 7.1-kb MscI-XhoI fragment, *mlc-thyl*, which contains *mlcR* genomic fragments flanking the *thyl* gene (Fig. 1) was purified and was used to transform JH10 cells. Transformation of *Dictyostelium* was performed as described by Howard et al. (1988). The thymidine-auxotrophic cell line, JH10, was electroporated with *mlc-thyl* and cells were selected in HL-5 media without exogenously added thymidine. Following the appearance of colonies, around the eighth day, cells from these colonies were transferred to six-well plates (Costar Corp., Cambridge, MA), and screened for the absence of RMLC by western blotting. Cells were further cloned by picking isolated plaques after growth on bacterial plates. Three independent RMLC null cell lines were confirmed by Southern blot analysis.

Dictyostelium RMLC cDNA (Tafari et al., 1989) was introduced into an integrating expression vector pBORP (Ostrow et al., 1994) to generate pBVN519. Expression of the RMLC is driven by the *Dictyostelium* actin 15 promoter (see Pollenz et al., 1992, for an example). Both pBORP and pBVN519 were then used to transform RMLC null cells to examine functional complementation of *mlcR*⁻ disruption.

Western Blot Analysis

Cells were harvested from plates and lysed in SDS sample buffer (0.125 M

Tris, pH 6.8, 4% SDS, 10% BME). Proteins were sized on 10% (for MHC) or 15% (for RMLC and EMLC) SDS-PAGE, blotted to nitrocellulose filter, and probed with a polyclonal anti-myosin antibody (NU-3) (Chisholm et al., 1988) or anti-LMM (kindly provided by Dr. A. DeLozanne, Duke University), followed by ¹²⁵I-labeled protein A as previously described (Pollenz et al., 1992).

Fluorescence Microscopy

Monoclonal antibodies specific for *Dictyostelium* MHC were the generous gift of Dr. Y. Fukui (Northwestern University) (Yumura et al., 1984), and Dr. G. Gerisch (Max Planck, Martinsreid, Germany) (Pagh and Gerisch, 1986). Actin antibody HB80 was also provided by Dr. Fukui. Cells were harvested and allowed to attach to coverslips for 10 min, then fixed and stained using the method described by Fukui et al. (1987), except that the agar-overlay step was eliminated. Myosin, actin staining and receptor capping assays were performed as described (Fukui et al., 1990). DAPI staining was performed according to Pollenz et al. (1992), except that cells were fixed with 1% formaldehyde in methanol for 4 min at -15°C. Photographs were taken through a Zeiss Axioscope or a laser scanning confocal microscope, using 63 or 100× objectives and TMAX100 film (Kodak).

Cell Aggregation and Development

Cells were harvested in the mid-log phase of growth and washed three times with buffered salt solution (Wessels et al., 1988). To initiate development, 5 × 10⁶ cells in 0.5 ml buffered salt solution were dispersed onto 2% agar. Development was observed at 0.5- to 1-h intervals for the first 15 h. Development was also observed on *Klebsiella aerogenes* lawns on SM agar plates (Sussman, 1987).

Biochemical Analysis

Myosin was purified from *Dictyostelium* cells as described by Pollenz et al. (Pollenz et al., 1992), except that ammonium sulfate precipitation step was omitted and a Superose 6 FPLC column (Pharmacia LKB Nuclear, Gaithersburg, MD) was used instead of S-500 column. Protein concentrations were determined by Bradford assay (Bradford, 1976). Cytoskeletons were prepared following procedures of Kuczmarski et al. (1991). The final pellets were suspended directly into Ca²⁺-ATPase reaction buffer (20 mM Tris-Cl, pH 8.0, 0.5 M KCl, 10 mM CaCl₂) and ATPase activity was determined according to Pollenz et al. (1992).

Myosin assembly assays were performed as described by Kuczmarski, et al., (1987). Purified myosin (stored in 0.5 M KCl, 10 mM Triethanolamine, pH 7.0, 1 mM EDTA, 1 mM DTT, and 1 mM NaN₃) was diluted into the desired salt concentration, kept on ice for 30 min, and centrifuged in a Beckman Airfuge at 23 Psi for 15 min. For disassembly, myosin was first assembled at 50 mM KCl and pelleted by centrifugation. The pellets were then resuspended in the desired salt conditions. In both cases, protein concentration of the supernatant was measured by Bradford assay.

Negative Stain Transmission Electron Microscopy

Myosin assembly was induced by rapid dilution of purified myosin in storage buffer to give a final myosin concentration of 0.1 mM in 10 mM triethanolamine, pH 7.0, 50 mM KCl, 10 mM MgCl₂, 0.1 mM DTT. Myosin filaments were then applied to carbon-over-formvar copper grids after varying periods of incubation. After 30 s, the grids were rinsed with two drops of 2.5% Uranyl Acetate at 1.5-min intervals. Excess stain was removed by touching the edge of the grid with a strip of filter paper. The grids were then air-dried before EM visualization on JEM-100CX.

Results

Disruption of the RMLC (*mlcR*) Gene

The thymidine-auxotrophic *Dictyostelium* cell line, JH10 (Mann and Firtel, 1991; Hadwiger and Firtel, 1992), was transformed with a gene targeting vector (Fig. 1A) designed to favor a double-crossover, gene replacement event. The linear DNA used was composed of 2.4-kb 5' and 1.7-kb 3' RMLC genomic sequence, with the 3-kb *thyI* gene (Dynes and Firtel, 1989) inserted into the second codon. After transformation, cells were selected in HL-5 medium lacking added thymidine and individual clones were then assayed for the expression of RMLC by Western blot, using a polyclonal *Dictyostelium* myosin specific antibody NU-3 (Fig. 2A). In four independent transformations, the percentage of clones without detectable RMLC expression ranged from 24–45%. Cell lines lacking RMLC were cloned by picking isolated

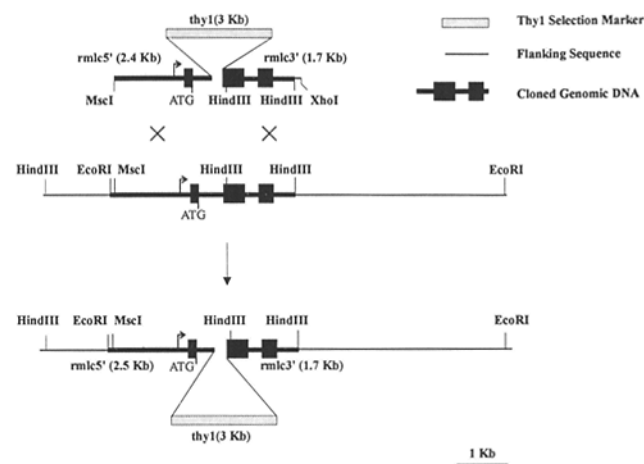
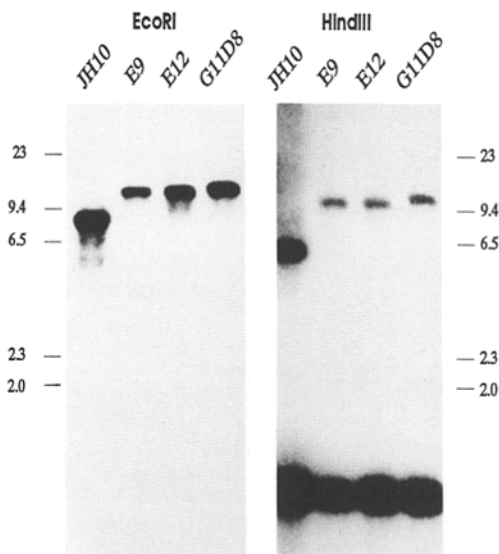


Figure 1. Generation of the *Dictyostelium* RMLC null cell lines. (A) Diagram of the gene replacement vector, the *Dictyostelium* *mlcR* locus, and the disrupted *mlcR* locus. Homologous recombination between the targeting vector and the *mlcR* gene locus will provide an *mlcR* gene carrying a 3-kb insertion in the second codon of the RMLC. (B) Genomic southern blot analysis of the *mlcR* locus in parental (JH10) and three different transformed cell lines (E12, E9, and G11D8). DNA was purified from parental JH10 cells and transformed cell lines (E9, E12, G11D8). Following digestion with restriction enzymes, DNA (4 mg) was loaded onto 0.7% agarose gel, electrophoresed, transferred to a nylon membrane, and hybridized with the ³²P-labeled RMLC cDNA. The left four lanes represent hybridization to genomic DNA digested with EcoRI; DNA in the right four lanes was digested with HindIII.



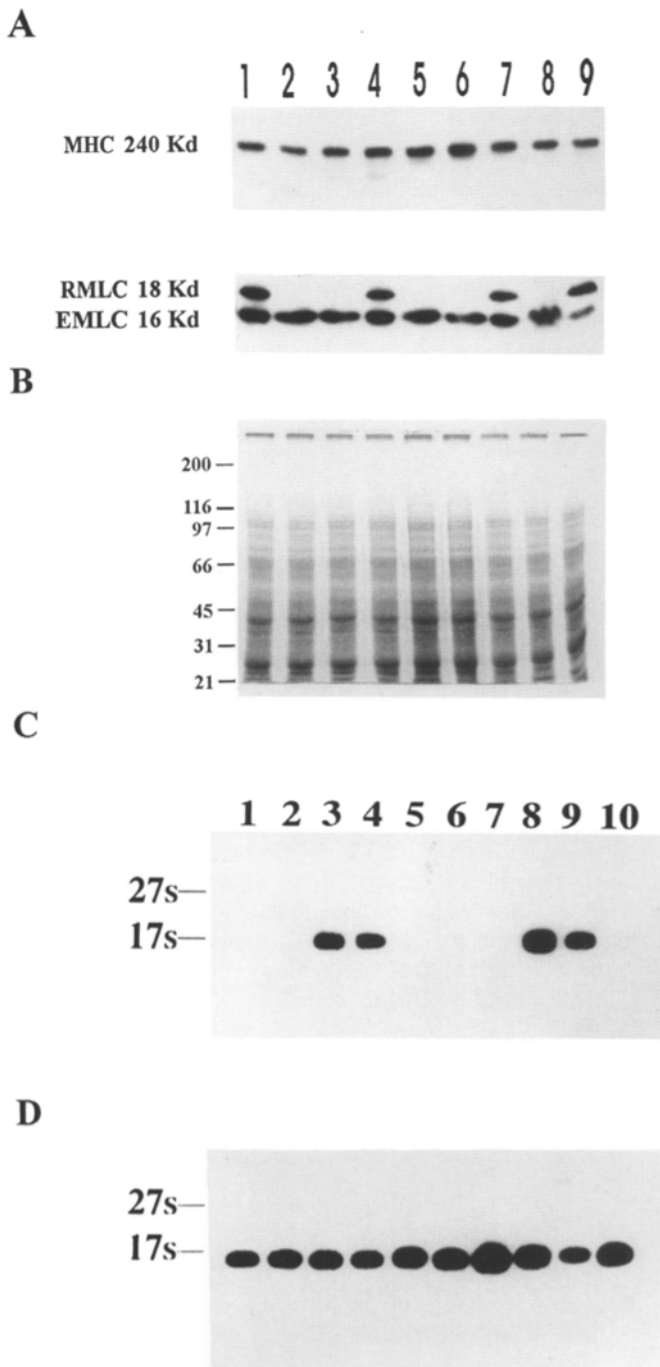


Figure 2. Expression of RMLC protein and mRNA in the *mlcR*⁻ and control *Dictyostelium* cell lines. (*A* and *B*) Western blot analysis of myosin heavy chain expression. 2×10^6 cells were lysed in SDS-PAGE sample buffer, separated in parallel on 10% (*A*, MHC) and 15% (*A*, RMLC and EMLC) polyacrylamide gels, transferred to nitrocellulose membranes. The blot for MHC was probed with anti-LMM polyclonal antibody to detect MHC expression. Polyclonal antibody NU-3 was used to detect RMLC and EMLC protein expression. Bound antibodies were detected with [¹²⁵I]protein A and subsequent autoradiography. *B* represents a 10% polyacrylamide gel run in parallel and stained in Coomassie Blue. Lane 1, parental JH10 cells; lane 2, RMLC null cell line E12; lane 3, E12 transformed with pBORP (E12pBORP); lane 4, E12 rescued with RMLC cDNA in pBORP (E12pRMLC); lane 5, RMLC null cell line E9; lane 6, E9 transformed with a *Dictyostelium* expression vector pBORP (E9pBORP); lane 7, E9 rescued with RMLC cDNA

plaques after growth on bacterial plates. Southern blot analysis was performed to determine the structure of *mlcR* gene locus in the RMLC-deficient cell lines. Genomic DNA was digested with EcoRI and HindIII separately, then hybridized with a probe made from RMLC cDNA fragment. This probe should hybridize to both the native *mlcR* locus and any site into which the exogenous gene replacement vector had inserted. The EcoRI fragment contains the entire *mlcR* gene, while HindIII cuts the DNA in the RMLC coding region, splitting the RMLC gene into 5' and 3' fragments. As expected for a double-crossover, homologous recombination event (Fig. 1 *B*), both the EcoRI fragment and the band representing the 5' HindIII fragment showed a size increase of ~ 3 kb, due to the insertion of *thyl* selection marker. The band corresponding to the 3' HindIII fragment did not vary in size.

Northern blots of *mlcR*⁻ cells probed with the RMLC cDNA showed no detectable ~ 800 nucleotide RMLC message (Fig. 2 *C*, lanes 1, 2, 5-7, and 10). The level of the EMLC mRNA in *mlcR*⁻ cells is comparable to that seen in wild-type cells (Fig. 2 *D*), suggesting that the level of the EMLC transcript is not altered significantly by the lack of RMLC expression.

To serve as a control in the analysis of the mutants, we introduced into the *mlcR*⁻ cells an integrating *Dictyostelium* expression vector pRMLC (Ostrow et al., manuscript submitted for publication), which restored RMLC expression at both the mRNA (Fig. 2 *C*, lanes 8 and 9) and protein level (Fig. 2 *B*, lanes 4 and 7). These rescued cell lines were phenotypically indistinguishable from wild-type cells in all subsequent assays.

Myosin heavy chain protein level in *mlcR*⁻ cells was assayed by western blotting of whole cell extracts, probed with anti-LMM polyclonal antibody kindly provided to us by Dr. DeLozanne (Duke University, Durham, NC) (Fig. 2 *A*). The level of MHC protein level does not vary significantly among *mlcR*⁻, wild-type parental and the rescued cell lines. Similar results were also obtained with a monoclonal anti-MHC antibody 396 (a generous gift from Dr. Gerisch, Max Planck, Martinsreid, Germany) (data not shown). This indicates that the phenotypic defects of *mlcR*⁻ cells cannot be attributed to a decrease in the amount of MHC in vivo.

Phenotypic Analysis of the *mlcR*⁻ Cells

mlcR⁻ cells are defective in cytokinesis. Although *mlcR*⁻ cells grew on plastic with nearly normal doubling time, when inoculated into suspension culture, they showed no increase in cell number over a 6-d period. Parallel suspension

in pBORP (E9pRMLC); lane 8, RMLC null cell line G11D8, lane 9, a non-homologous recombinant transformed cell line H11. (*C* and *D*) Northern blot analysis. RNA was harvested from vegetative *Dictyostelium* cells and loaded onto 1% agarose gel (10 μ g/lane), separated by electrophoresis, transferred to a nylon membrane, and probed with ³²P-labeled RMLC cDNA (*C*). After autoradiography, the nylon membrane was stripped of radioactivity and rehybridized with ³²P-labeled EMLC cDNA (*D*). Lane 1, E9pBORP; lane 2, E12pBORP; lane 3, parental cell line JH10; lane 4, H11 (non-homologous recombinant); lane 5, RMLC null cell line E9; lane 6, RMLC null cell line E12; lane 7, RMLC null cell line G11D8; lane 8, E9pRMLC; lane 9, E12pRMLC; lane 10, G11D8pBORP.

cultures of wild-type and rescued cell lines grew with a doubling time of 12 ± 2 h. Two days after introduction of the *mlcR*⁻ cells into suspension, many large multinucleate cells were observed. These cells were often at least 10 times the size of wild-type cells and frequently contained 20–30 nuclei per cell (Fig. 3 *a*). When the cells were returned to solid substrate, large multinucleate cells rapidly fragmented into several smaller cells (Fig. 3 *e*), perhaps via a process that has been termed traction-mediated cytofission (Fukui et al., 1990). Two or more cytofission events often occur at the same time with long cytoplasmic bridges connecting each pair of daughter cells (Fig. 3 *e*). The long bridges, formed by stretched plasma membrane, were frequently seen to extend 10–20 cell lengths before the connection broke. In contrast, wild-type cells showed normal cleavage furrows with relatively short mid-bodies (Fig. 3 *f*). *mlcR*⁻ cells rescued by the introduction of the RMLC expression construct exhibited normal cytokinesis and showed a normal distribution of one to two nuclei per cell when grown in suspension.

Myosin organization is abnormal in *mlcR*⁻ cells. Immunofluorescence confocal microscopy was used to examine myosin and actin localization in wild-type (Fig. 4, *a–c* and *m–o*) and *mlcR*⁻ cells both grown on plastic (plate-grown) (Fig. 4, *d–l*) and incubated in suspension for two days (Fig. 4, *p–r*). Cells were harvested and allowed to attach to coverslips for 10 min, then fixed and processed for immunofluorescence. Actin localization was primarily cortical with strong signals observed in cell surface projections in cells cultured in either suspension or on plates. In contrast, the pattern of myosin localization in *mlcR*⁻ cells varied depending upon culture condition (Compare Fig. 4, *d* or *g* to *p*). *mlcR*⁻ cells taken from suspension cultures display strong cytoplasmic myosin staining with very little cortical staining (Fig. 4 *p*). After attaching to the coverslips, suspension-cultured cells frequently initiate the process dubbed “traction-mediated cytofission.” Myosin failed to localize to the cytoplasmic bridges in these cells. Myosin staining was often observed as dense patches and rod-like

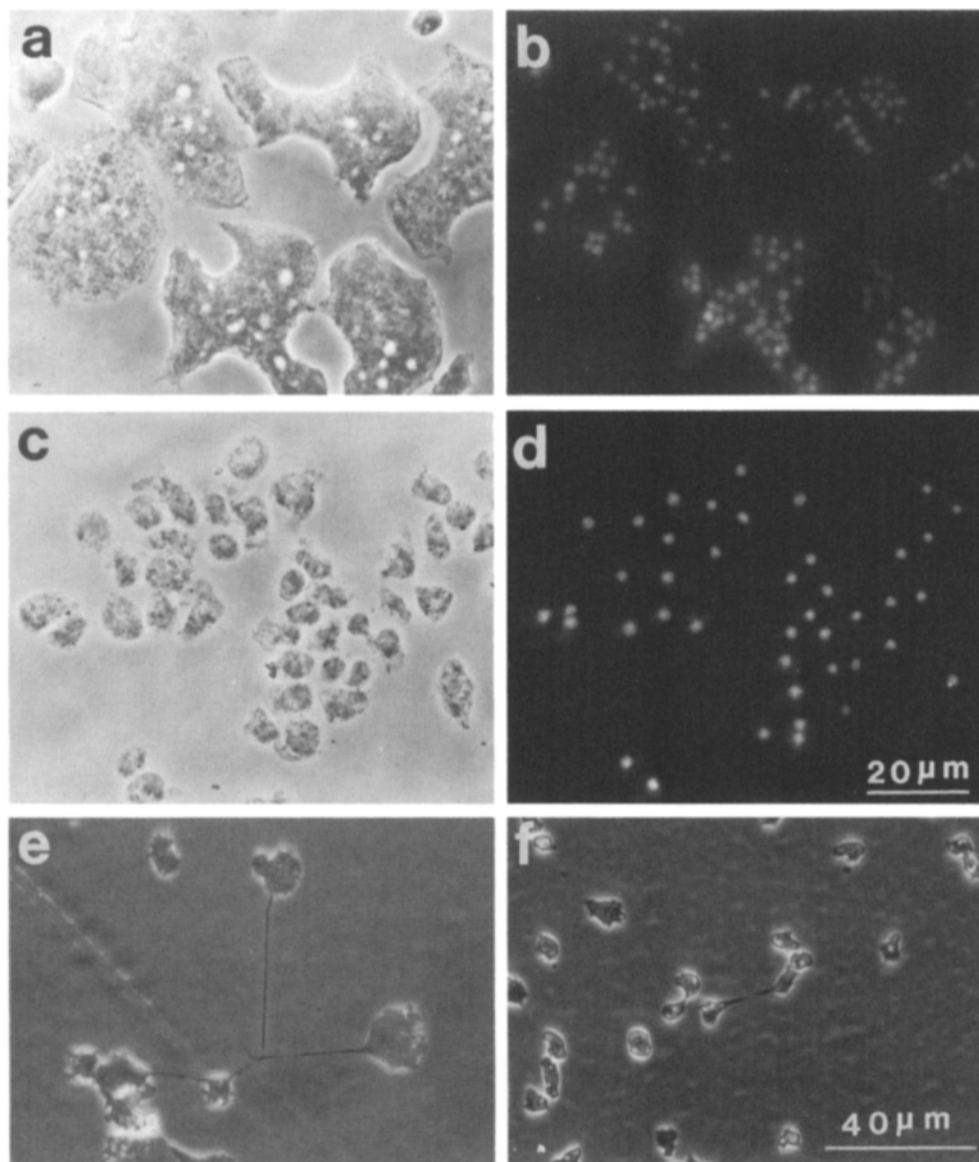


Figure 3. Cytokinesis is defective in RMLC null cells. (*a–d*) RMLC null (*a* and *b*), and cells rescued by introduction of an RMLC expression construct (*c* and *d*) were taken from the 2-d suspension cultures, allowed to attach to coverslips for 10 min and then examined by phase-contrast optics (*a* and *c*) and fluorescence microscopy after DAPI staining (*b* and *d*). Note the differences in the cell size and number of nuclei per cell in RMLC null and the rescued cells. (*e* and *f*) The difference in cytoplasmic division between suspension-cultured RMLC null cells (*e*) and parental cells (*f*) were also observed by phase-contrast microscopy. After they were transferred onto the coverslip from 2-d suspension culture, *mlcR*⁻ cells often formed long cytoplasmic bridges during cytoplasmic division, while such bridges are rarely seen in wild-type cells and they are much shorter than those observed in the *mlcR*⁻ mutants. Bars: (*d*) 20 μm; (*f*) 40 μm.

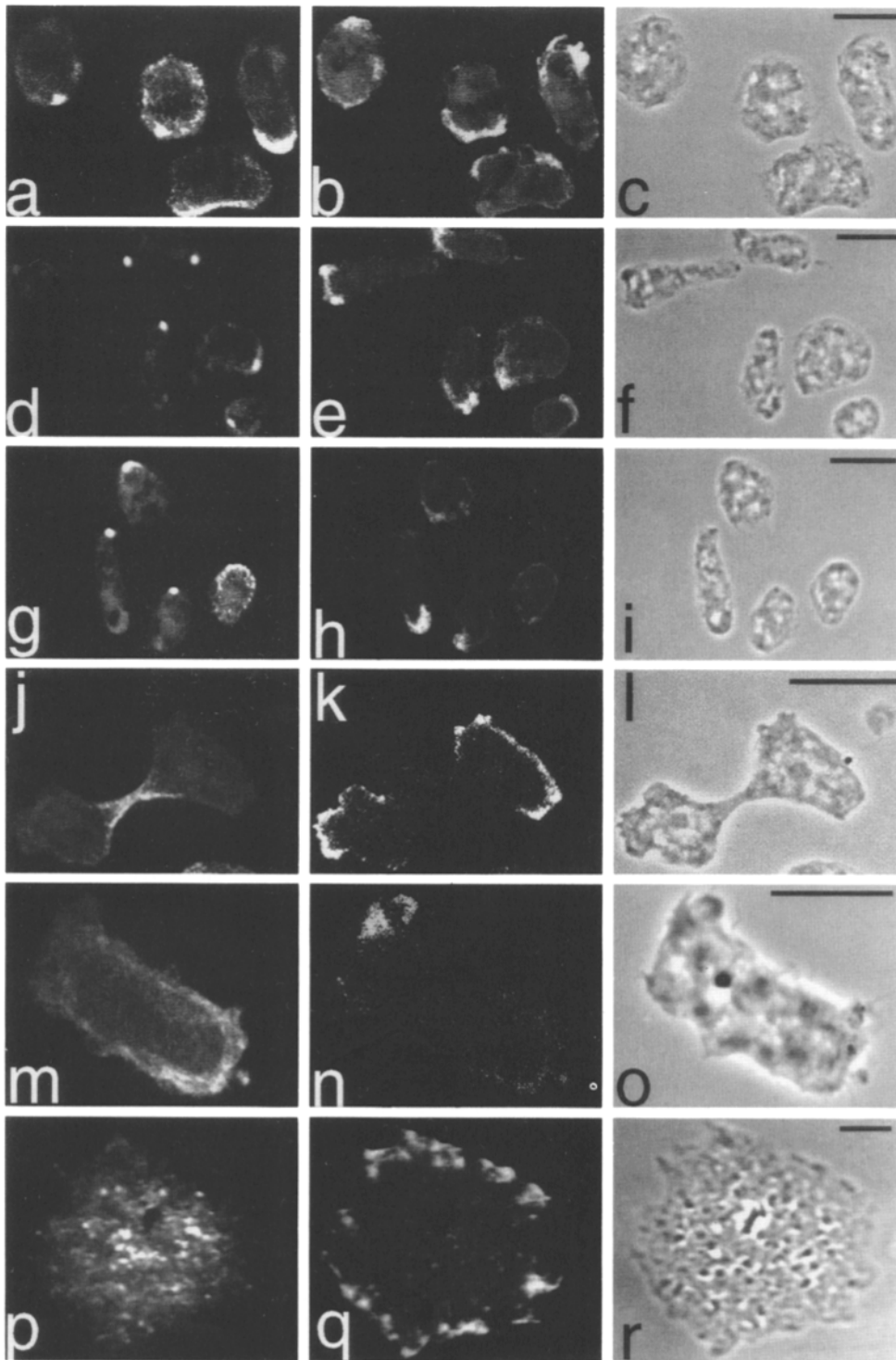


Figure 4. Myosin and actin localization in RMLC null cells. Myosin and actin localization of RMLC null cells was examined by immunofluorescence confocal microscopy and phase-contrast optics. Cells were allowed to attach to coverslips for 10 min before they were fixed and stained. (a-c) Myosin (a) and actin (b) localization in wild-type *Dictyostelium* cells following growth on plastic plates. Note the bright myosin staining distributed at the posterior region and actin staining at the leading edge of cells. (d-f) Myosin (d) and actin (e) staining of RMLC null cell E12 taken from plastic plates. Note the normal myosin staining pattern in some cells, and only a brightly stained spot at the posterior cortex in others (d). Actin staining is rich in the anterior, similar to wild-type cells. (g-i) Myosin (g) and actin (h) staining of RMLC null cell E9 grown on plastic plates. The staining patterns are similar to those in E12 cells. Some cells exhibit normal myosin staining pattern, while the rest show only an intense myosin spot at the rear end. Actin staining is seen in the leading edge of E9 cells. (j-l) Myosin (j) and actin (k) localization in a *mlcR*⁻ cell undergoing cytoplasmic division on a solid substrate. Note the proper localization of myosin to the cleavage furrow and actin to the leading edge of the cells. (m-o) Myosin (m) and actin (n) staining of wild-type JH10 cells taken from suspension cultures and allowed to attach to the coverslip for 10 min. Myosin and actin localization is the same to those in plate-grown JH10 cells. (p-r) Myosin (p) and actin (q) localization in RMLC null E12 cells taken from 2-d shaking culture and allowed to

attach to the coverslip for 10 min. The cells have many bright patches and rod-like structures in the endoplasmic region, yet failed to exhibit cortical myosin staining (p). However, actin staining is intense in cell surface projections (q). Bars, 10 μ m.

structures distributed throughout the endoplasm. Plate-grown *mlcR*⁻ cells, on the other hand, often showed normal cortical myosin staining (Fig. 4, d and g) as well as localization to an apparent cleavage furrow when cells were going through cell division (Fig. 4 j). However, a significant percentage of plate-grown *mlcR*⁻ cells (40–80% depending on

the cell line) exhibited discrete spots of intense myosin staining at or close to the cortex (Fig. 4, d and g). Myosin localization in wild-type cells is always more extensively distributed along the cell cortex (Fig. 4 a). No cell was observed to have more than one spot. The “myosin spot” was most often localized to the posterior end of the cells. Cells with this

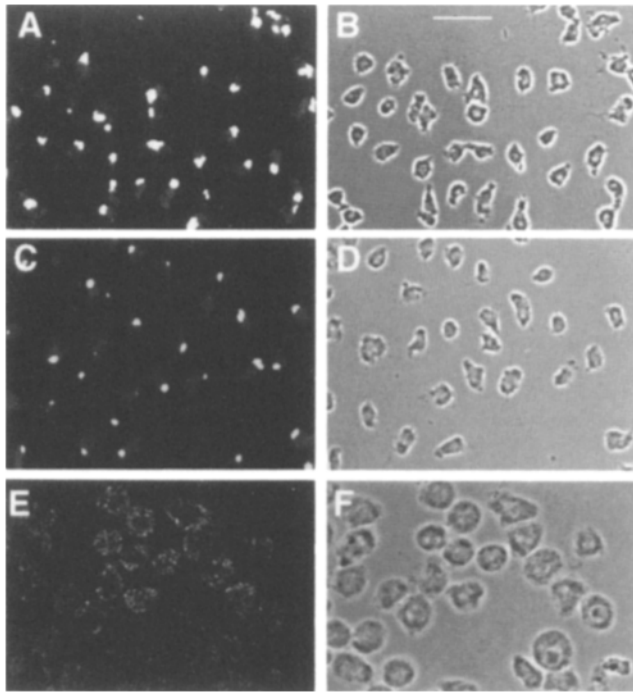


Figure 5. RMLC null cells can complete receptor capping. Fluorescein-labeled con A was incubated with wild-type JH10 (A and B), RMLC null (C and D), and MHC null (E and F) cells for 20 min. Fluorescently labeled caps indistinguishable from wild type started to appear within 10 min in RMLC null cells (C). In MHC null cells, only patches but not caps of fluorescence were observed (E). Bar, 20 μ m.

“myosin spot” usually did not exhibit significant myosin staining elsewhere, although a few cells had both the myosin spot and normal cortical staining.

mlcR⁻ cells can form con A caps. When *Dictyostelium* cells lacking MHC (Pasternak et al., 1989; Fukui et al., 1990) or the EMLC (Pollenz et al., 1992) were treated with FITC-con A, they were unable to cap their cell surface receptors. As can be seen in Fig. 5, mlcR⁻ cells capped their receptors when exposed to FITC-con A under conditions where the MHC null cells failed to do so. The time course of cap appearance was not distinguishable from that of wild-type controls under the conditions we used.

mlcR⁻ cells cannot complete their development cycle. Upon starvation, mlcR⁻ cells and the parental line JH10 cells both aggregated on hydrophobic agar. JH10 cells and rescued cells started aggregation after 6–8 h, formed tight aggregates after 8–11 h and fruiting bodies after 24–26 h. mlcR⁻ cells aggregated more slowly than wild-type cells, aggregating after 8–10 h, forming tight aggregates after 12–14 h and never proceeded beyond the formation of mounds (Fig. 6). Migrating slugs or fruiting bodies were never seen.

Purified RMLC⁻ myosin forms abnormal aggregates after assembly. Myosin was purified from mlcR⁻ cells using a standard protocol for *Dictyostelium* myosin (Clarke and Spudich, 1974). Based on SDS-PAGE/Western blot analysis, amounts of RMLC⁻ myosin comparable to wild type were recovered at each step before the removal of actin by gel filtration. Following gel filtration, the myosin-containing

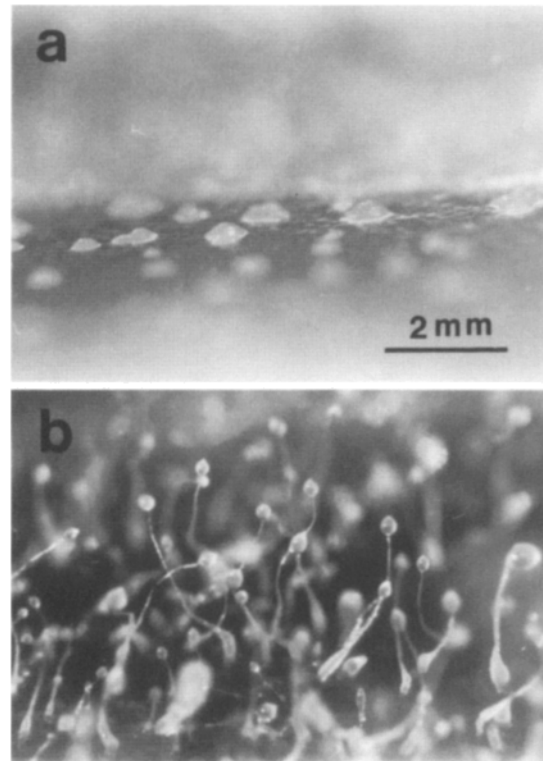


Figure 6. RMLC null cells failed to complete development. *Dictyostelium* cells in HL-5 were inoculated onto freshly spread lawns of *Klebsiella* on SM plates. Plates were photographed after 6 d. Development of RMLC null cells was arrested at the “mound” stage (a), while rescued cells completed development (b). Bar, 2 mm.

fractions were assembled in 50 mM KCl, pelleted and resuspended in 500 mM KCl buffer. After this step, in seven independent experiments, the resulting yield of myosin was typically about one fifth the amount obtained from the JH10 parental cell line, although yields at prior steps were comparable. This solubilized myosin we assayed never had more than 10% the actin-activated ATPase (10 + 7 nmol Pi/min/mg myosin) or 5% the calcium-ATPase activity (26 + 8 nmol Pi/min/mg myosin) of wild-type myosin (actin-activated ATPase: 147 + 40 nmol Pi/min/mg myosin; Ca²⁺-ATPase: 800 + 91 nmol Pi/min/mg myosin). To obtain a crude estimate of the in vivo activity of the mutant myosin, we assayed the Ca²⁺-ATPase in cytoskeletons pre-

Table I. Ca²⁺-ATPase Activity of Triton-insoluble Cytoskeletons

Cell Line	Ca ²⁺ -ATPase
	nmol Pi/mg/min
Wild type (JH10)	7.29 ± 0.30
45 E12 (mlcR ⁻)	2.64 ± 0.23
43 E9 (mlcR ⁻)	2.2 ± 0.99
MHC null (mhcA ⁻)	0.38 ± 0.37

Cytoskeletons were prepared from the different cell lines indicated as described by Kuczmarzski et al. (1991). The final pellets were resuspended directly into Ca²⁺-ATPase reaction buffer (20 mM Tris-Cl, pH 8.0, 0.5 mM KCl, 10 mM CaCl₂) and ATPase was measured according to Pollenz et al. (1992).

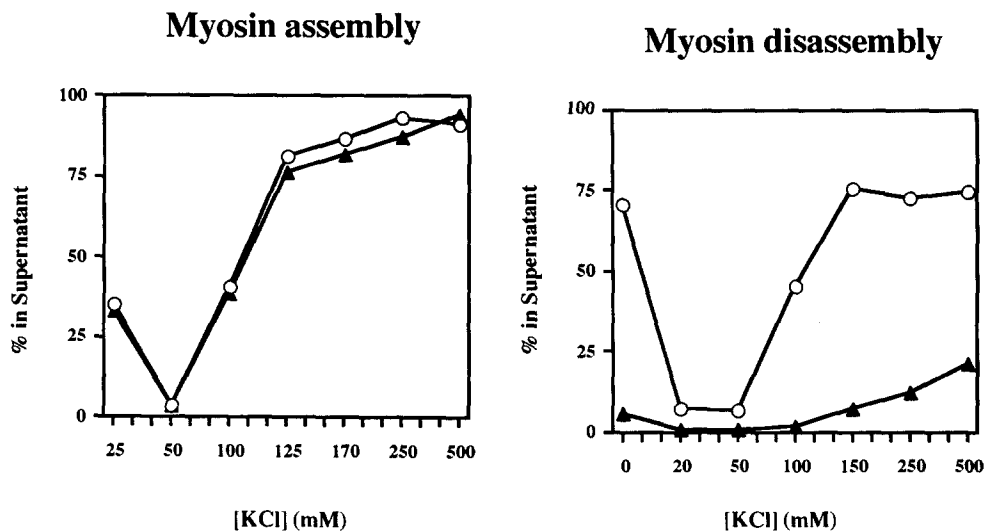


Figure 7. Assembly and disassembly of RMLC⁻ myosin. (A) Salt-dependent assembly: wild-type (open circles) and RMLC⁻ (solid triangles) *Dictyostelium* myosin was assayed for assembly by centrifugation at the varying KCl concentrations. The amount of protein in the supernatant was measured. The assembly of RMLC⁻ myosin has essentially the same salt dependence as that of wild-type myosin. (B) Salt-dependent disassembly: assembled wild-type (open circles) and RMLC⁻ (solid triangles) *Dictyostelium* myosin was pelleted by centrifugation, resuspended at the indicated KCl concentration, and pelleted again in the airfuge. RMLC⁻ myosin disassembles very poorly at all KCl concentrations tested.

pared from wild-type, RMLC null, and MHC null cells. As can be seen from Table I, cytoskeletons from *mlcR*⁻ RMLC null cells had ~40% the ATPase activity of wild-type cytoskeletons, while cytoskeletons from MHC null cells essentially had no activity. Based on Western blot analysis, *mlcR*⁻ cytoskeleton preparations contained amounts of myosin comparable to that of wild-type cytoskeletons. Although it is impossible to obtain precise activity measurements in these crude preparations, it is clear that RMLC⁻ cytoskeletons had Ca²⁺-ATPase activity significantly higher than cytoskeletons from MHC null cells, but lower than wild type. It should be noted that Ca²⁺-ATPase is a measurement of the ability of the myosin head to hydrolyze nucleotide, but is not physiologically relevant. Since the active site appears to be intact *in vivo* but has reduced activity when purified, these results suggest that RMLC⁻ myosin ATPase activity is less stable during purification than wild-type myosin.

Because of the reduced yield observed following the final assembly of the RMLC⁻ myosin, we examined the assembly and disassembly properties of the purified RMLC⁻ myosin, using an ultracentrifugation assay (Kuczmarski et al., 1987). As can be seen in Fig. 7 A, RMLC⁻ myosin displayed a profile of salt-dependent assembly similar to wild-type myosin. In contrast, once assembled, the RMLC⁻ myosin failed to disassemble efficiently at the salt concentrations assayed (Fig. 7 B). This suggested that the structures formed after the RMLC⁻ myosin assembly might be abnormal.

We examined negative stained preparations of assembled wild-type and RMLC⁻ myosin by electron microscopy.

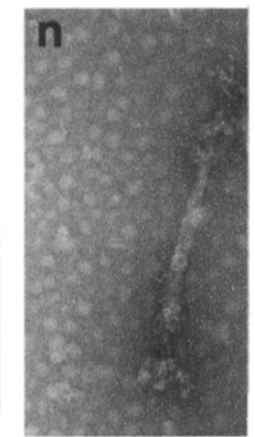
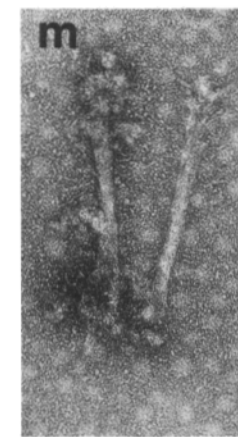
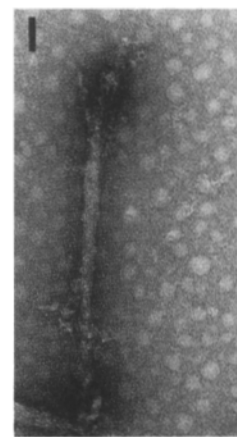
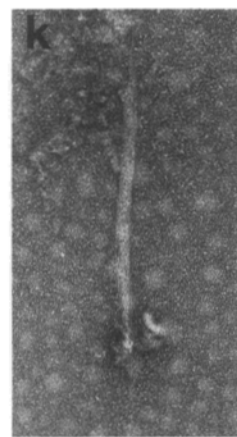
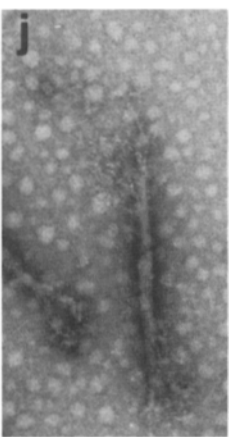
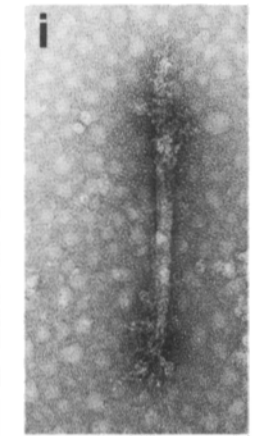
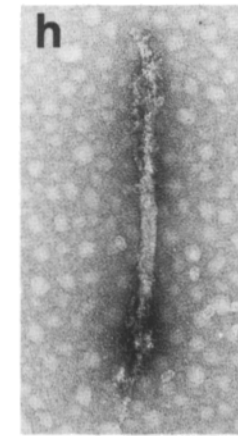
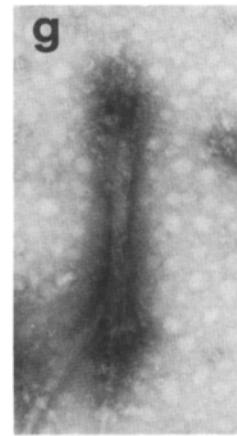
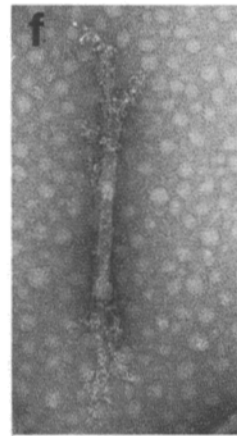
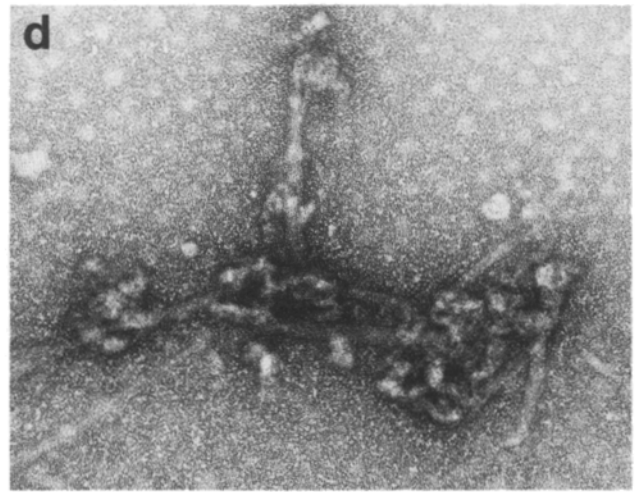
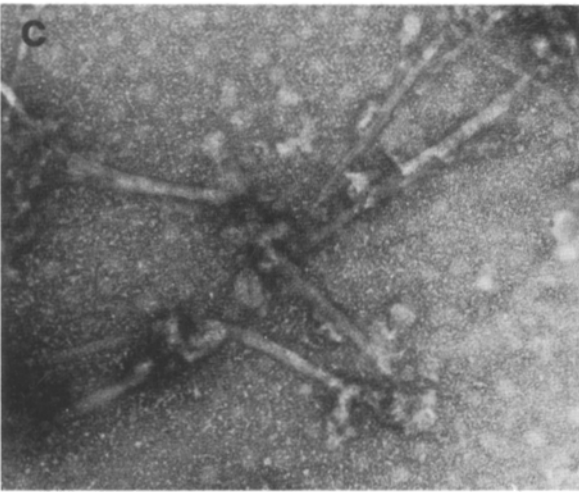
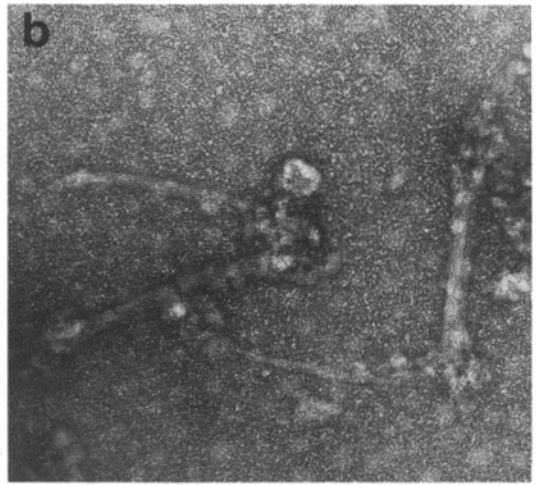
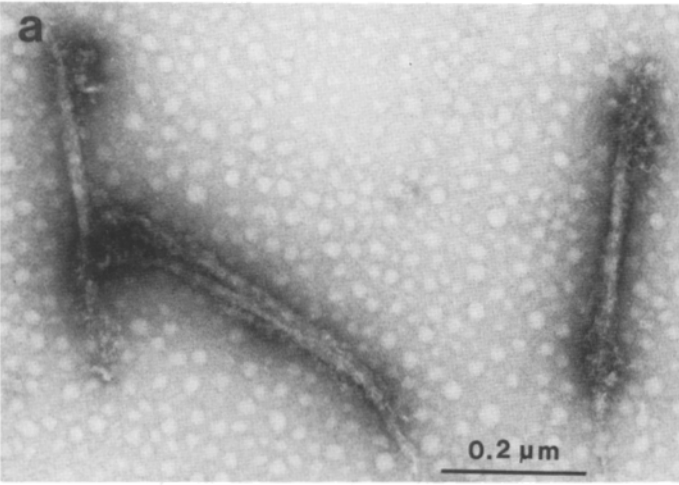
When samples were taken after only 2 min of assembly, RMLC⁻ myosin formed thick filaments (Fig. 8, *j-n*) similar to those observed with wild-type myosin (Fig. 8, *e-i*). However, by 10 min of assembly, few isolated filaments were seen in preparations of RMLC⁻ myosin. Instead, filaments appeared to associate, often at their ends, forming large, amorphous aggregates (Fig. 8, *b-c*). By 30 min of assembly, only these aggregates were observed in RMLC⁻ myosin (Fig. 8 *d*), while wild-type myosin remained largely isolated filaments (Fig. 8 *a*).

Discussion

We have used targeted disruption of the *mlcR* gene to produce *Dictyostelium* cells that fail to express regulatory myosin light chain. Southern blot analysis of these cells indicated that a single copy of the transforming vector was inserted at the *mlcR* gene locus. Northern and Western blot analysis showed that no RMLC mRNA or polypeptide was expressed in the *mlcR*⁻ cells. Despite the absence of RMLC, the level of MHC and EMLC polypeptides was unchanged in these cells, providing additional evidence for the lack of a feedback mechanism to coordinate the expression of myosin subunits (Pollenz et al., 1992).

Phenotypically the *mlcR*⁻ cells resemble the *mhcA*⁻ cells in several ways, including defective cytokinesis in suspension and failure to complete the developmental program (DeLozanne and Spudich, 1987; Wessels et al., 1988; Manstein et al., 1989; Fukui et al., 1990). When cultured in suspension *mlcR*⁻ cells failed to divide and became large and

Figure 8. Structure of RMLC⁻ myosin filaments assembled *in vitro*. (a) wild-type *Dictyostelium* myosin after assembly on ice for 30 min. (b-d) RMLC⁻ myosin after assembly for 2 (b), 10 (c), or 30 min (d). Increased assembly time leads to increased numbers of aggregated structures compared to wild-type myosin which seldom forms amorphous aggregates. (e-n) gallery of isolated wild-type (e-i) or RMLC⁻ (j-n) myosin filaments. The *mlcR*⁻ myosin was only assembled for 2 min while the wild type was assembled for 30 min. Bar, 0.2 μm.



multinucleate. *mlcR*⁻ cells also failed to progress beyond the mound stage during multicellular development. *mlcR*⁻ cells also share the phenotypic defect in cytokinesis with *spaghetti-squash*, the *Drosophila* cytoplasmic RMLC mutant (Karess et al., 1991) and *Dictyostelium* cells expressing reduced EMLC (7-11) as a consequence of overexpression of antisense EMLC mRNA (Pollenz et al., 1992). Thus, at least with respect to these processes, the RMLC is required for normal myosin function in vivo.

However, unlike the *mhcA*⁻ cells, the *mlcR*⁻ are able to cap their cell surface receptors following cross-linking by con A. Since *mhcA*⁻ cells fail to cap receptors, this process is believed to be myosin dependent (Pasternak et al., 1989; Fukui et al., 1990). The fact that *mlcR*⁻ cells cap receptors suggests that, in vivo, myosin lacking the RMLC retains the activity or activities necessary for this process. In contrast, cells expressing reduced EMLC (7-11) (Pollenz et al., 1992) and *mlcR*⁻ cells differ in their ability to cap surface receptors. 7-11 cells are unable to cap receptors while *mlcR*⁻ cells appear capable of normal capping.

Our observation that myosin is localized abnormally in both suspension- and plate-grown cells may provide one clue to explain the loss of myosin function in *mlcR*⁻ cells. In suspension-grown *mlcR*⁻ cells, the myosin appears to be found in a more cytoplasmic rather than cortical location. In addition, the pattern of myosin staining is more punctate than that observed in wild-type cells, which appears more filamentous, suggesting that some *mlcR*⁻ myosin may be in abnormal aggregates instead of normal thick filaments. It is unlikely that the abnormal myosin localization observed in suspension cultured cells is a nonspecific consequence of their large multinucleate morphology. *Dictyostelium* cells which have a similar large, multinucleate morphology due to a greatly reduced expression of EMLC show normal cortical distribution of myosin (Pollenz et al., 1992). In addition, Kitanishi-Yumura and Fukui (1989) have induced large multinucleate *Dictyostelium* cells by treatment with microtubule poisons and observed normal localization of myosin to cleavage furrows that formed upon removal of the drug. The idea that inappropriate myosin localization contributes to the phenotypic defects in the *mlcR*⁻ cells is also supported by the unusual disassembly properties of the myosin purified from these cells. Although initial assembly of filaments by monomeric *mlcR*⁻ myosin appears normal, the assembled myosin appears to rapidly form aggregates probably by association at the head/rod junction as seen in RMLC⁻ smooth and skeletal myosins (Wagner and Giniger, 1981; Trybus and Lowey, 1988; Trybus et al., 1994). The aggregates formed no longer exhibit the disassembly properties of normal thick filaments and they may correspond to the apparent patches observed in suspension-cultured *mlcR*⁻ cells, as well as the intense spot of myosin staining observed in plate-grown *mlcR*⁻ cells.

The pattern of myosin localization in plate-grown *mlcR*⁻ cells is quite different from the suspension-grown *mlcR*⁻ cells, but not completely normal. Many cells (between 20 and 60%) show a normal pattern of cortical localization. In addition, cells undergoing cytoplasmic division on plates were all observed to have myosin localized properly to their cleavage furrows. However, between 40–80% of the *mlcR*⁻ cells were observed to have a single, intense spot of myosin staining. The nature of this spot is unknown. Based on ap-

pearance and localization, the myosin spot we see in the *mlcR*⁻ cells may be similar to those found in cells transformed with a mutant myosin heavy chain that lacks the assembly regulatory domain (Δ C34) found in the COOH-terminal 34-kD domain of the heavy chain (Egelhoff et al., 1991). These heavy chain mutants are defective in their ability to disassemble once they have formed thick filaments. It is possible that the accumulation of myosin in the spot-like structures may sequester myosin, effectively removing myosin from its normal location. The spots could, for example, represent a site where abnormal myosin aggregates are targeted for turnover. However, since cells expressing mutant Δ C34 heavy chain are capable of cytokinesis in suspension and can complete the developmental program, the absence of RMLC results in a more severe deficiency in myosin function than the Δ C34 heavy chain mutant. In Δ C34 heavy chain-expressing cells, the myosin is 80% Triton-insoluble, compared with 13% for wild type, suggesting that much of the myosin in these cells may be unable to disassemble and thereby reducing contractile activity at sites where it is normally found. Because the defect in *mlcR*⁻ cells is much more severe than Δ C34 expressing cells, and 20–60% of the *mlcR*⁻ cells have normal patterns of myosin distribution, it seems unlikely that the defects resulting from the absence of RMLC is due exclusively to improper myosin localization or inability of the *mlcR*⁻ myosin to disassemble.

Alternatively, the intense "myosin spot" could represent a transient structure involved in normal myosin function in vivo. In this scenario, the absence of the RMLC would either stabilize this structure or increase the length of time myosin remains associated with it. The observation of only a single spot per cell and its consistent posterior cortical location strengthens the notion that the spot is a specific structure rather than a random myosin aggregate. *mlcR*⁻ cells without the myosin spot could represent cells which have lost this structure, especially during cytoplasmic division. Further experiments are necessary to characterize the nature of this myosin spot, and to understand the lack of cortical localization of myosin in suspension-cultured cells.

Biochemically, *mlcR*⁻ myosin exhibits two abnormal properties. First its enzymatic activity is significantly more labile than is that of wild-type myosin. The absence of significant Ca²⁺-ATPase activity raises the possibility that the protein might be significantly denatured during the purification process despite the fact that wild-type myosin was stable throughout the purification. Second, although purified *mlcR*⁻ myosin assembles into reasonable thick filaments in vitro, the structure of these filaments rapidly degenerates, forming amorphous aggregates which no longer show KCl-dependent disassembly. This abnormal disassembly of RMLC⁻ myosin may also provide at least a partial explanation for our inability to isolate enzymatically active RMLC⁻ myosin, because in vitro assays of myosin ATPase activity are performed under conditions where the myosin is expected to be filamentous. Both smooth and skeletal muscle myosin from which the light chains have been removed in vitro have been shown to retain reasonable ATPase activities (Wagner and Giniger, 1981; Trybus and Lowey, 1988; Trybus et al., 1994). Lowey et al. (1993) have recently shown that skeletal muscle myosin from which both light chains have been removed in vitro has significantly reduced motor activity despite the fact that it retains, at least

briefly, ATPase activity $\sim 50\%$ that of myosin reconstituted with both light chains. Following removal of the RMLC by trifluoperazine treatment, smooth muscle myosin shows increased ATPase activity (Trybus et al., 1994). Assuming that *Dictyostelium* myosin behaves in a similar fashion, it is likely that *mlcR*⁻ myosin would have reasonable ATPase in vivo, at least for a while after synthesis, but that the length of time required to purify the myosin exceeds the length of time during which it is enzymatically active. Indeed, our crude assay of Ca²⁺-ATPase activity in cytoskeletons showed levels of activity as high as 40% of wild type in *mlcR*⁻ cytoskeletons, while *mhcA*⁻ cytoskeletons showed only a few percent the wild-type activity. This result suggests that the active site of RMLC⁻ myosin is structurally intact, although we have no evidence of physiologically relevant actin-activated ATPase in vivo. However, it is clear from these studies that myosin lacking the RMLC is enzymatically less stable during purification than is myosin carrying the RMLC. Hence it may have a decreased half life and therefore decreased overall activity in vivo.

It has been proposed that the light chains may amplify the movement resulting from ATP hydrolysis, effectively increasing the distance a myosin filament could move relative to actin for each ATP hydrolyzed. The recent data from Lowey et al. (1993), Uyeda and Spudich (1993), and Trybus et al. (1994) support this hypothesis. Myosin motor activity, as assayed by the rates of actin filament movement in an in vitro motility assay are reduced in myosins lacking the RMLC. The reduced ability of *mlcR*⁻ cells to complete the contraction of the cleavage furrow during cytokinesis, and the inability to complete development, may be in vivo consequences of this possible role for the RMLC in force generation. If so, the ability of *mlcR*⁻ cells to cap cell surface receptors suggests either that receptor capping requires significantly less force generation, or that cytokinesis and morphogenesis are dependent on different aspects of myosin function than is receptor capping. It is interesting to note that EMLC-deficient cells which had normal myosin localization pattern failed to cap surface receptors (Pollenz et al., 1992).

The results presented here suggest that the defects observed in *mlcR*⁻ cells may arise from any of several different mechanisms or a combination of them. The first is the improper myosin localization resulting from aggregation of myosin which may exist in an abnormal assembly state. This would have the effect of reducing function by preventing myosin from accumulating at sites where it would normally provide contractile activity. A second mechanism might be decreased enzymatic stability. Based on the three dimensional structure proposed by Rayment et al. (1993), removal of the RMLC would expose an α -helical region of the neck. Without the protection provided by the RMLC, this region may become denatured, affecting the overall structure of the myosin head, or it may facilitate the aggregation of myosin through association of the hydrophobic residues exposed by the absence of the RMLC. Third, myosin lacking the RMLC may be less efficient at generating force, due to a decreased effective step size resulting from increased flexibility of the neck region. Finally, we cannot eliminate the possibility that the RMLC has some additional function distinct from its role with myosin II, although there is no indication that such an alternative function exists.

As recently demonstrated by Uyeda and Spudich (1993),

myosin lacking the domain of the MHC required for RMLC binding (and therefore lacking the RMLC), rescued the cytokinesis defect of *mhcA*⁻ cells, and at least partially restored multicellular development. The results reported here argue that the RMLC provides a significant function in the presence of wild-type MHC. If this function does not provide a selective advantage, it seems likely that myosin heavy chain mutations removing the RMLC binding site and its requirement for the RMLC would have occurred during evolution. In addition to possible roles in amplifying the conformational changes of the myosin head and stabilizing the enzymatic activity, the RMLC is also thought to be an important regulatory component of myosin and its regulatory function may provide an evolutionary advantage. The *mlcR*⁻ cells generated in these studies should prove valuable in future studies of the mechanism of myosin-linked regulation, serving as a host into which mutant light chains can be introduced and the in vivo consequences of those mutations characterized.

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