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Myosin light chain kinase (MLCK) gene disruption in *Dictyostelium*: A role for MLCK-A in cytokinesis and evidence for multiple MLCKs

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ABSTRACT **We have created a strain of *Dictyostelium* that is deficient for the Ca^{2+}/calmodulin-independent MLCK-A. This strain undergoes cytokinesis less efficiently than wild type, which results in an increased frequency of multinucleate cells when grown in suspension. The MLCK-A⁻ cells are able, however, to undergo development and to cap crosslinked surface receptors, processes that require myosin heavy chain. Phosphorylated regulatory light chain (RLC) is still present in MLCK-A⁻ cells, indicating that *Dictyostelium* has one or more additional protein kinases capable of phosphorylating RLC. Concanavalin A treatment was found to induce phosphorylation of essentially all of the RLC in wild-type cells, but RLC phosphorylation levels in MLCK-A⁻ cells are unaffected by concanavalin A. Thus MLCK-A is regulated separately from the other MLCK(s) in the cell.**

The molecular motor myosin II (referred to throughout as "myosin") was originally studied as the force-generating protein in muscle, but it is also found in a wide variety of nonmuscle cells, including the slime mold *Dictyostelium discoideum. Dictyostelium* that lack functional myosin have been generated using gene disruption or antisense RNA techniques (1–6). These cells are motile and able to form aggregates upon starvation, but the aggregates do not develop into fruiting bodies. *Dictyostelium* lacking the myosin heavy chain or essential light chain also cannot cap surface proteins that have been crosslinked with concanavalin A (Con A; refs. 4 and 7). Finally, myosin⁻ cells are unable to undergo cytokinesis in suspension, where they become large and multinucleate, and eventually lyse. These cells can be maintained on a surface, where they divide by a myosin-independent mechanism (1).

It is well established that actin and myosin are a major part of the cellular machinery for cytokinesis (8, 9). Less, however, is known about the regulation of this process (10). Myosin from muscle and nonmuscle cells is activated by phosphorylation of the regulatory light chain (RLC) by MLCK (11–14). It has been proposed that RLC phosphorylation may play an important role in myosin regulation during cytokinesis (9, 15, 16).

While MLCKs are traditionally thought to require Ca^{2+}/calmodulin for activity, in *Dictyostelium* a calmodulin-independent MLCK (MLCK-A) has been purified and its gene (*mlkA*) cloned (17–19). This 34-kDa enzyme consists of a catalytic domain followed by a 39 amino acid inhibitory domain. Like other known MLCKs, it is very specific for myosin light chains (17, 18). *In vivo* MLCK-A is activated by phosphorylation in the activation loop (20).

We have undertaken a molecular genetic study of this calmodulin-independent MLCK in *Dictyostelium discoideum*, because this organism exhibits a number of myosin-dependent behaviors, and is amenable to study by a wide variety of techniques. We have found that *Dictyostelium* contains more than one MLCK and that MLCK-A is required for efficient cytokinesis, but is not required for multicellular development. ConA treatment of cells was found to induce phosphorylation of the RLC in wild-type cells, but not in MLCK-A⁻ cells, indicating that MLCK-A is regulated separately from the other MLCK(s) in the cell.

METHODS

Media. For normal axenic growth, either HL5 (21) or a 3:1 mixture of HL5 and FM (GIBCO/BRL) was used. Heat-killed *Klebsiella aerogenes* was prepared by resuspending the cells from a 100-ml stationary-phase culture in 1 ml of 10 mM sodium phosphate buffer (pH 7.5), and heating in a boiling water bath for 15 min. This was used as a 50× supplement to HL5 and was stored at 4°C for up to 3 days. 2-(*N*-Morpholino)ethanesulfonic acid (Mes) starvation buffer was prepared as described in Berlot *et al.* (22). For metabolic labeling, phosphate-free FM was prepared by substituting 20 mM Mes (pH 6.8) for phosphate in the FM recipe described in Franke and Kessin (23).

Strains. JH10 and DH1 have been described (24, 25). JH10 is referred to as "wild type" in this paper. HS183, described below, is JH10 with the *mlkA* gene disrupted. This strain is referred to as MLCK-A⁻ in this paper.

PCR of *mlkA* from Genomic DNA. The two oligonucleotides 5′-CCGGATCCATGACAGAAGTAGAAAAAATATACG (MLCK-5′) and 5′-CCGGATCCTTAGTTAACCAATTTT-GTTTGAGTT (MLCK-3′B) were synthesized at the Beckman Center Protein and Nucleic Acid Facility (Stanford University). Both contain a *Bam*HI site near their 5′ end. In MLCK-5′ the *Bam*HI site is followed by 28 residues corresponding to the very 5′ end of the coding sequence of the *mlkA* cDNA (sense strand), and in MLCK-3′B the *Bam*HI site is followed by 28 residues corresponding to the very 3′ end of the coding sequence of the *mlkA* cDNA (antisense strand). PCR mixture contained 500 nM each MLCK-5′ and MLCK-3′B, 10 μg/ml Ax-2 genomic DNA, 0.25 unit/μl native *Taq* DNA polymerase (Perkin–Elmer/Cetus), 200 μM each dNTP, 4 mM $MgCl_2$, 10 mM Tris·HCl (pH 8.3), and 50 mM KCl. Amplification was performed in a Perkin–Elmer Cetus DNA Thermal Cycler under the following cycle conditions: five cycles at 94°C for 1 min, 37°C for 1 min, and 72°C for 2 min, followed by 15 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min.

***mlkA* Gene Disruption.** The DNA construct used for the gene disruption was created as follows. First the *Sac*I site was removed from the cloning vector pGEM2 (Promega) by removing the 60-bp *Hin*cII–*Pvu*II fragment. This plasmid was designated pGEM2ΔHP. The genomic *mlkA* PCR product

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Abbreviations: MLCK, myosin light chain kinase; RLC, regulatory light chain; DAPI, 4′,6-diamidino-2-phenylindole.

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described above was digested with *Bam*HI and cloned into the *Bam*HI site of pGEM2ΔHP to give pJS16. The *thy1* gene (24) was then cloned as a 3.3-kb *Hinc*II fragment from pLS21 into the *Pvu*II site of pJS16, such that the reading frame was in the opposite direction as the open reading frame from *mlkA*. This plasmid, which was designated pJS18, was linearized with *Sac*I, and 5 μg were electroporated into JH10 (26). The electroporated cells were split into three 10-cm Petri plates and grown in HL5. Ten days later, three or four isolated colonies from each plate were transferred to 24-well plates. Once the cells had formed a confluent layer, they were resuspended, diluted in Mes starvation buffer, and spotted onto SM/5 plates with pregrown lawns of *K. aerogenes* (21). Isolated fruiting bodies were used to inoculate HL5 (containing penicillin and streptomycin to kill any residual bacteria) in 24-well plates. Once they had formed a confluent layer, these cells were plated on SM/5 plates with *K. aerogenes*. The resulting lawns of fruiting bodies were resuspended in 15% glycerol and frozen at −80°C. Fresh cultures were started from these frozen stocks every 4–6 weeks.

4′,6-Diamidino-2-phenylindole (DAPI) Staining. Cells that had been passed for less than two weeks in HL5 were diluted into HL5 supplemented with heat-killed *K. aerogenes*, and grown for 15–17 hr to a density of $1–5 \times 10^6$/ml. Concentrated EDTA was added to an aliquot of cells to a final concentration of 5 mM, and the suspension was mixed by gently pipetting up and down three times with a micropipettor to reduce clumping. For collecting data on the number of nuclei per cell, the cells were then fixed by mixing with an equal volume of HL5 plus 2% formaldehyde and left at room temperature for 10 min. The cells were then gently pelleted and resuspended in Mes starvation buffer with 0.2 μg/ml DAPI. After 10 min, the cells were viewed by fluorescence microscopy. To decrease the background staining from the *K. aerogenes* for the fluorescent micrographs in Fig. 1, the cultures were washed free of *K. aerogenes* and then grown for ≈2 hr in HL5. Cells (50 μl at 1×10^6/ml) were allowed to attach to a coverslip for 15 min and were then fixed in 1% formaldehyde in PBS for 10 min, followed by acetone (−8 to −15°C) for 5 min. The coverslips were rinsed once in PBS and mounted in 10% glycerol containing 0.2 μg of DAPI per ml.

Protein Purification and Production of Antibodies. *Escherichia coli* cells expressing RLC with six histidine residues at its N-terminus (His-RLC) were kindly provided by Rex Chisholm (Northwestern University, Chicago). Soluble His-RLC was purified on a Ni^{2+}-column as described by the manufacturer (Qiagen, Chatsworth, CA), using 5 mM imidazole to wash the column and 200 mM imidazole to elute. MLCK-A was expressed in *E. coli* and purified as described (20).

Rabbit sera directed against RLC and MLCK-A were prepared at Berkeley Antibody (Richmond, CA). One rabbit was initially immunized with 500 μg purified recombinant His-RLC, and two rabbits were each initially immunized with 500 μg purified recombinant MLCK-A. Boosts of 250 μg of purified protein were performed at 3-week intervals, and the rabbits were exsanguinated 11 days after the third boost. All antibodies were affinity purified before use. Purified protein was coupled to CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer's protocol, and antibodies were purified as described in Harlow and Lane (27), except that only the acid elution of the column was performed.

Metabolic Labeling and Immunoprecipitation of ^{32}P-Labeled RLC. Cells (5–10 ml) growing at $1.5–6.7 \times 10^6$/ml in either HL5 or a 1:1 mix of HL5 and FM were harvested, washed once in phosphate-free FM, and resuspended in 10 ml of phosphate-free FM at $1.2–3.5 \times 10^6$ cells/ml. The cells were shaken at 300 rpm at 22°C. After 7 hr of phosphate starvation, 10^7 cells were concentrated to 2×10^7/ml by harvesting them by centrifugation, and removing the medium to leave a final volume of 0.5 ml. The cell pellets were resuspended, and 200-μl aliquots were transferred to 1.5-ml screw-cap tubes (Starstedt, Newton, NC). [^{32}P]P$_i$ (ICN; 100 μCi; 1 Ci = 37 GBq) was

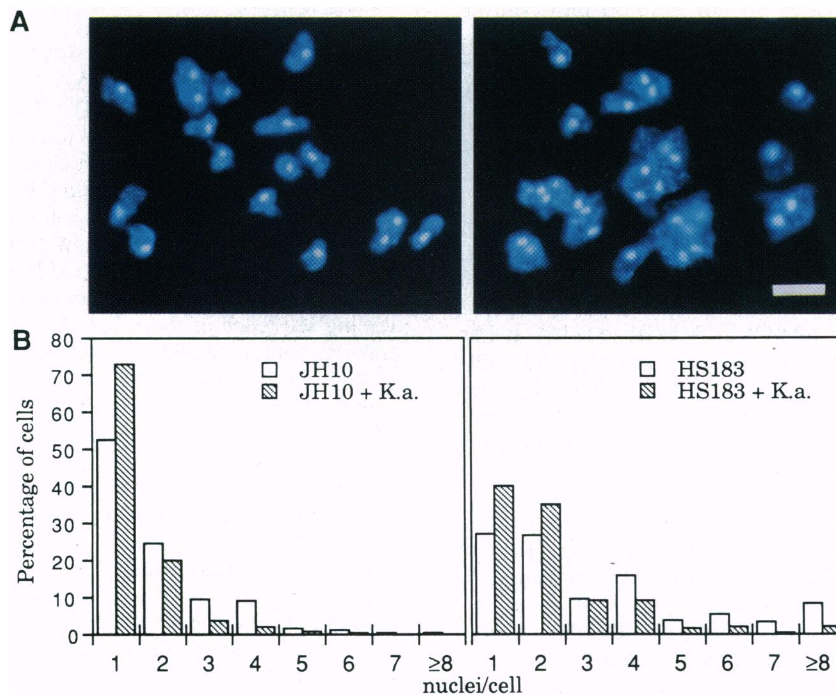

FIG. 1. Quantitation of the number of nuclei in wild-type and MLCK-A⁻ cells. (*A*) DAPI staining of JH10 (wild-type) and HS183 (*mlkA*⁻) cells. Cells grown in suspension were briefly allowed to adhere to coverslips, then fixed and stained. Left, JH10; right, HS183. (Bar = 20 μm.) (*B*) The number of nuclei per cell was determined by DAPI staining of cells that had been fixed in suspension. Data were pooled from five separate experiments. For growth with *K. aerogenes*, 816 JH10 cells and 815 HS183 cells were scored. For axenic growth, 698 JH10 cells and 695 HS183 cells were scored. Similar results were obtained with HS181, an independently isolated disruption of *mlkA* in JH10. The difference between the two strains diminished with prolonged passage; cultures which had been passed for less than 2 weeks were used for these studies.

added to each tube, and the cultures were rotated end-over-end for 3 hr at 22°C. The labeled cells were harvested for 2 min in a Microfuge at 2000 × *g*, washed in Ca^{2+}-free Mes starvation buffer, and resuspended in 180 μl of 20 mM Mes, pH 6.8/20 mM NaCl/1 mM EDTA. Twenty microliters of 10% SDS was added to lyse the cells, and the lysates were immediately boiled for 15 min. Four microliters of Pansorbin (Calbiochem) was added to promote the formation of a tight pellet, and the lysates were clarified by centrifugation for 5 min at top speed in a Microfuge. The supernatant was carefully removed and transferred to a fresh tube containing 1 ml of IP dilution buffer (28), 20 μl of 1:1 protein A Sepharose slurry (Sigma), and 10 μl of affinity-purified His-RLC antibody, then rotated end-over-end overnight at 4°C. The immunoprecipitation complexes were washed as described in Rothblatt and Schekman (28).

Con A Treatment of Cells. JH10 and HS183 cells were grown to log phase in HL-5 medium. For each sample, 3×10^6 cells were allowed to settle on the surface of a 60-mm Petri dish for 5 min. Adherent cells were washed once with Ca^{2+}-free Mes starvation buffer, then incubated with 2.5 ml of the same buffer with or without 30 μg/ml Con A (Sigma) for 4 min. After incubation, cells were quickly resuspended with a micropipettor. One milliliter of the suspension was removed to a tube containing 100 μl of 100% (wt/vol) trichloroacetic acid. The precipitated cells were collected by centrifugation and resuspended in 2× urea sample buffer (9.1 M urea/21 mM Trizma base/192 mM glycine/1.5 mM EDTA/1 mM dithiothreitol.)

The samples were resolved by electrophoresis on a glycerol gel, after the method of Perrie and Perry (29) with the following modifications (J. L. Tan, personal communication). A stacking gel of 3.5% acrylamide, 0.13% bisacrylamide, and 71 mM Tris·HCl (pH 6.8) was used, and the separating gel was 7.5% acrylamide, 0.28% bisacrylamide, 20 mM Trizma base, 177 mM glycine, and 50% (wt/vol) glycerol. The gel was run at 3 W for 3 hr in 20 mM Trizma base/177 mM glycine. The RLC bands were visualized by immunoblotting using a monoclonal antibody against myosin RLC (30) and an enhanced chemiluminescence detection kit (Amersham).

For microscopy of Con A-induced capping of cells, the concentration of cells grown in HL5 was adjusted to 1×10^6 cells/ml. A drop of cells was placed within a circle drawn on a coverslip with a hydrophobic pen. Cells were allowed to settle on the coverslip for 5 min. The adhered cells were incubated in Mes starvation buffer containing 50 μg/ml tetramethylrhodamine-conjugated Con A (Sigma) for 1 or 5 min. The cells were fixed in 2% formaldehyde in Mes starvation buffer for 10 min, then rinsed in starvation buffer and water. Coverslips were mounted in 10% glycerol and photographed with a fluorescence microscope.

Azide Treatment of Cells. Cells grown in HL5 (5×10^6) were allowed to adhere to a 100-mm Petri dish for 10 min. The media was replaced with HL5 containing 2 mM NaN_3, and detachment of cells after 5 min was monitored with an inverted microscope.

RESULTS

Creation of a MLCK-A Null Mutant. Early attempts to disrupt the *mlkA* gene using constructs derived from cDNA clones of the locus were unsuccessful. We suspected that the gene might contain introns that split up the homology into ineffective pieces, and we characterized the genomic locus to determine whether this was the case. PCR primers to the 5′ and 3′ ends of the *mlkA* coding region were used to generate a genomic copy of the gene. While the coding region deduced from the cDNA sequence is 0.9 kb in length (19), the PCR product was 1.8 kb, indicating that there is 0.9 kb of intron DNA embedded within the 0.9 kb of coding DNA. There could also be additional intron DNA at the very 5′ or 3′ ends of the gene that is spanned by the PCR primers, and therefore not recovered in this PCR fragment. Comparison of the restriction maps of the genomic PCR and cDNA clones showed that *mlkA* has at least four introns distributed throughout the coding region. Most likely the cDNA-based constructs did not work because they lacked sufficiently long stretches of homology with the genomic locus to promote homologous recombination.

The *mlkA* locus was disrupted in the thymidine auxotroph JH10 (26) by targeting it with a construct containing the *thy1* gene (24) inserted near the middle of the MLCK-A gene. *Thy1* transformants were selected based on their ability to grow in media that was not supplemented with thymidine. Of 17 colonies screened by immunoblot analysis, 10 lacked the MLCK-A protein. Cloned cells from two of these colonies (HS183 and HS181) became our working MLCK-A$^-$ strains. Replacement of the *mlkA* gene with the *thy1*-disrupted allele was confirmed by Southern blot analysis (data not shown).

Characterization of the MLCK-A$^-$ Cells. Since MLCK-A activates myosin contractile activity, we tested whether the phenotype of MLCK-A$^-$ cells resembled the phenotype of cells lacking functional myosin. Unlike myosin$^-$ cells, the MLCK-A$^-$ cells divided in shaking culture, indicating that these cells are not completely deficient for cytokinesis. The doubling times of wild-type and MLCK-A$^-$ cells were indistinguishable (5.7 ± 0.3 hr for JH10 and 5.7 ± 0.6 hr for HS183 in HL5 supplemented with heat-killed *K. aerogenes*). However, MLCK-A$^-$ cells were on average larger than wild-type cells, and we showed that most were multinucleate by DAPI staining (Fig. 1*A*). Since both the wild-type parent strain JH10 and the MLCK-A$^-$ mutant HS183, when grown axenically, had a high percentage of multinucleate cells (Fig. 1*B*, stippled bars), we also grew cells in media supplemented with heat-killed *K. aerogenes*, which greatly reduces the number of multinucleate cells (T. Q. P. Uyeda, personal communication). A pronounced difference between wild-type and mutant was evident both in cultures grown axenically and in cultures that were supplemented with heat-killed *K. aerogenes* (Fig. 1*B*). In cultures containing a high percentage of multinucleate cells (i.e., JH10 when grown axenically or HS183 grown either axenically or in the presence of heat-killed *K. aerogenes*), multinucleate cells containing an even number of nuclei were slightly more common than those containing an odd number of nuclei. In cultures grown with heat-killed bacteria, the average number of nuclei per cell for JH10 cells was 1.4, with 27% multinucleate cells, whereas the average number of nuclei per cell for the MLCKA$^-$ strain HS183 cells was 2.2, and 60% were multinucleate. In cultures grown axenically, the average number of nuclei per cell for wild-type cells was 1.9, with 47% multinucleate cells, whereas the average number of nuclei per cell for the HS183 cells was 3.3, and 73% were multinucleate. We have also disrupted the *mlkA* gene in DH1 (25), an unrelated *Dictyostelium* strain. This parent strain, when grown axenically, had an average of 1.4 nuclei per cell, and 34% multinucleated cells, whereas the MLCK-A$^-$ cells derived from this strain had an average of 1.8 nuclei per cell, and 53% of the cells were multinucleate. Thus although MLCK-A is not required for cytokinesis, it does facilitate it.

The MLCK-A$^-$ cells were tested for several other functions known to be impaired in myosin$^-$ cells. Under starvation conditions, myosin$^-$ cells are able to aggregate, but are unable to complete multicellular development and form fruiting bodies. In contrast, MLCK-A$^-$ cells were able to complete development, at a pace identical to that of wild type (data not shown). Also, MLCK-A$^-$ cells could cap membrane proteins that had been crosslinked by the tetravalent lectin Con A, a process that requires functional myosin (Fig. 2). Finally whereas myosin$^-$ cells remain attached to a surface upon brief azide treatment (7, 31), adherent MLCK-A$^-$ cells, like wild type, rounded up and dissociated from the surface upon treatment with azide.

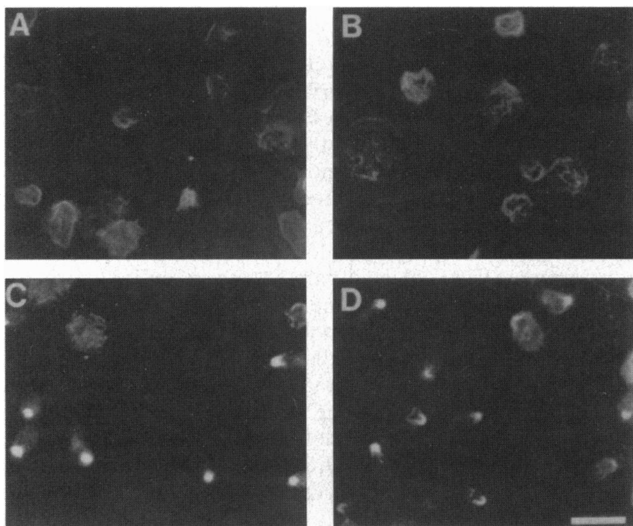

FIG. 2. Con A induces capping of cell surface receptors in both wild-type and MLCK-A$^-$ cells. (*A*) JH10 cells (wild-type), and (*B*) HS183 (*mlkA*$^-$) treated with tetramethylrhodamine-conjugated Con A for 1 min (*C*) JH10 cells, and (*D*) HS183 treated with tetramethylrhodamine-conjugated Con A for 5 min. (Bar = 20 μm.)

Phosphorylated RLC Is Found in Both Wild-Type and MLCK-A$^-$ Cells. Since the defects in MLCK-A$^-$ cells are less severe than myosin$^-$ cells, we suspected that the cells may possess a redundant kinase activity. To determine whether there is any phosphorylated RLC in the MLCK-A$^-$ cells, cultures were metabolically labeled with [^{32}P]P$_i$, and the RLC was immunoprecipitated. As a control, a strain lacking the RLC was also labeled (5). Even though MLCK-A is not present in the HS183 cells, ^{32}P-labeled RLC was recovered from these cells, as well from the JH10 parent cells (Fig. 3). Thus there is an additional kinase capable of phosphorylating the RLC. This additional kinase could either be a dedicated MLCK, or a protein kinase with a broader specificity range. MLCK-A phosphorylates the RLC on Ser-13 *in vitro*, and a Ser-13 $\rightarrow$ Ala mutant RLC is not detectably phosphorylated *in vivo*, suggesting that Ser-13 is the only phosphorylation site, and that the additional kinase would also phosphorylate the RLC on Ser-13 (32).

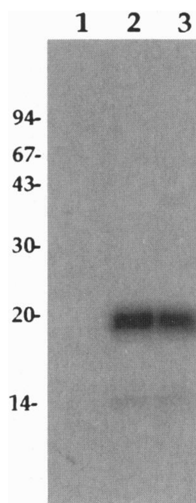

FIG. 3. Light chain phosphorylation in the MLCK-A$^-$ cells. Cells were metabolically labeled with [^{32}P]P$_i$, and the RLC was immunoprecipitated under conditions that dissociate it from the myosin heavy chain. Lane 1, RLC$^-$ cells; lane 2, JH10 cells; lane 3, HS183 (*mlkA*$^-$) cells.

RLC Is Phosphorylated in Response to Con A in Wild-Type but Not MLCK-A$^-$ Cells. We wanted to see if RLC phosphorylation is induced when cells are activated to undergo myosin-dependent processes, and, if so, whether MLCK-A is responsible. We did not see substantial changes in the level of RLC phopshorylation upon treatment of cells with azide, or during development (data not shown). Since capping of cell surface receptors requires functional myosin, we decided to see whether the level of RLC phosphorylation changes upon treatment of cells with Con A. We electrophoresed whole-cell lysates on a urea-glycerol gel (29), which separates unphosphorylated and phosphorylated RLC, and then detected the RLC bands by immunoblotting. We found that in wild-type cells, RLC phosphorylation increases from $\approx$50% to 100% after treatment with Con A (Fig. 4, lanes 1 and 2). In the MLCK-A$^-$ cells, the initial level of RLC phosphorylation is similar, but no increase is observed upon Con A treatment (Fig. 4, lanes 3 and 4). Thus RLC phosphorylation is induced in response to Con A, and MLCK-A is required for this induction. This experiment also reconfirms the existence of an additional MLCK in *Dictyostelium* and demonstrates that the different MLCKs are regulated separately.

DISCUSSION

The Role of RLC Phosphorylation and MLCK-A in *Dictyostelium*. *In vitro*, RLC phosphorylation of *Dictyostelium* myosin has been shown to result in a 6-fold increase in steady-state ATPase activity (17). This degree of regulation is rather modest compared with that observed with smooth muscle myosin, although steady-state measurements do typically underestimate the level of activation (see, for example, ref. 33). A more dramatic effect on *in vitro* motility has been reported: only 0.9% of beads coated with unphosphorylated myosin move on actin cables, whereas 75% of phosphorylated beads moved (17). In the sliding filament assay, myosin which is $\approx$10% phosphorylated shows slower, jerky movement similar to that observed with a low density of fully phosphorylated myosin (T. Q. P. Uyeda, personal communication.) So although the effect of RLC phosphorylation on *Dictyostelium* myosin needs to be characterized in more detail, the available data suggests that it is likely to be quite important for myosin function *in vivo*. In addition, RLC phosphorylation is regulated during chemotaxis (22, 34), and we have shown here that it is

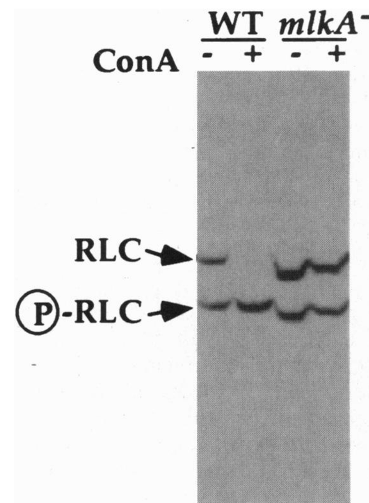

FIG. 4. ConA induces RLC phosphorylation. Wild-type (JH10) and *mlkA*$^-$ (HS183) cells were treated with Con A, and whole cell lysates were electrophoresed on a urea glycerol gel to resolve the phosphorylated and unphosphorylated forms of the RLC. The RLC bands were visualized by immunoblotting with a RLC antibody.

induced during Con A capping. These findings indicate that MLCKs are involved in regulating myosin-dependent cellular functions in *Dictyostelium*.

We have found that the phenotype of MLCK-A$^-$ cells is related to the phenotype of myosin$^-$ cells, but is less severe. Our observation that MLCK-A$^-$ cells are multinucleate indicates that MLCK-A is needed for efficient cytokinesis, but the fact that the cells grow in suspension shows that it is not essential for this process. In contrast, myosin$^-$ cells are completely unable to undergo cytokinesis in suspension. The other myosin-dependent processes tested, development, capping, and rounding up in response to azide, were unaffected in the MLCK-A$^-$ cells. It may be that unphosphorylated RLC is sufficient for these processes, and that the ability to assemble and disassemble filaments, together with the reduced contractile activity of unphosphorylated myosin, suffices. There is still, however, phosphorylated RLC in the MLCK-A$^-$ cells, so it is possible that some or all of these processes do require phosphorylated myosin, but that the phosphorylated RLC still found in the MLCK-A$^-$ cells is sufficient.

In this study we have investigated the *in vivo* role of RLC phosphorylation by eliminating one of the MLCKs in the cell. In a related study, Ostrow *et al.* (32) investigated the *in vivo* role of phosphorylation by introducing a modified RLC with an alanine at Ser-13, the phosphorylation site. They found that this mutant RLC can substitute for the wild-type RLC *in vivo*. This surprising finding implies that myosin need not be activated by RLC phosphorylation to function *in vivo*. Further studies will be required to reconcile this finding with our observation that cytokinesis is impaired in MLCK-A$^-$ cells. Regardless of how essential the increase in myosin activity by RLC phosphorylation proves to be, it almost certainly enhances the ability of *Dictyostelium* to compete in the wild. Moreover, the study of RLC phosphorylation by MLCKs provides an important inroad into understanding signaling during the various myosin-dependent processes in the cell.

MLCK-A and Capping. We have observed that capping of crosslinked surface proteins is associated with phosphorylation of essentially all of the RLC in the cell. This induction of RLC phosphorylation does not occur in the MLCK-A$^-$ cells, which strongly suggests that MLCK-A carries out this phosphorylation. While MLCK-A has been shown to phosphorylate myosin *in vitro,* the activity reported was surprisingly low (17–19). For this reason, and because it is not Ca^{2+}/calmodulin activated, which has been the classical hallmark of MLCKs, it was especially encouraging to find that this kinase does indeed function as a MLCK *in vivo*. We have recently found that the activity of MLCK-A is greatly enhanced by phosphorylation in the activation loop by an upstream kinase (20). The low activity observed in the previous studies is thus attributed to the fact that these preparations lacked this activating modification.

Although capping is associated with a dramatic increase in RLC phosphorylation, this increase is not required, since the MLCK-A$^-$ cells still are able to cap. RLC phosphorylation might alter the rate or some other parameter of capping in a manner that is too subtle to be measured in our experiments. Since capping is myosin-dependent, myosin phosphorylation may occur as a specific part of this process. However, it is also possible that RLC phosphorylation is induced as a result of Con A-induced crosslinking of receptors that activate MLCK-A. Con A is known to fortuitously activate receptors in other systems (reviewed in ref. 35). The role of myosin in capping in *Dictyostelium* is reminiscent of the finding that myosin and actin localize with adherent enteropathogenic *E. coli*, which is also accompanied by phosphorylation of the myosin RLC (36).

Multiple MLCKs in *Dictyostelium*. Our observation that *Dictyostelium* has more than one MLCK is likely to be related to the fact that myosin has multiple roles in these cells. Myosin is required for cytokinesis, development, and capping, and also plays a role in cell locomotion and chemotaxis. This is a diverse group of cellular processes, each of which is presumably regulated by distinct signaling pathways. Since the activation of myosin contractile activity by RLC phosphorylation is likely to be a part of some, if not all, of these diverse myosin-dependent processes, it is not surprising that there would be multiple MLCKs. Similarly, myosin heavy chain phosphorylation in *Dictyostelium*, which causes myosin filament disassembly, is carried out by multiple myosin heavy chain kinases (37–42). Heavy and light chain phosphorylation of myosin, together with the wide variety of mechanisms regulating actin (reviewed in ref. 43), undoubtedly provide a rich system for the temporal and spatial regulation of the actomyosin cytoskeleton.

To date Ca^{2+}/calmodulin-dependent MLCKs have been identified in several types of mammalian nonmuscle cells (44, 45), and calmodulin-independent MLCKs have been identified in *Physarum* (46) and *Dictyostelium* (17–19). In addition, it has recently been shown that mitogen-activated protein kinase-activated protein (MAPKAP) kinase 2 (47) and rho-associated kinase (48) can phosphorylate myosin on the same site as MLCK. It is likely that most organisms have multiple MLCKs, including one that is similar to MLCK-A. Investigators may have previously attributed any calmodulin-independent activities to proteolysis, which is known to render MLCKs calmodulin-independent. Of the kinases that have been sequenced thus far, the catalytic domain of MLCK-A shows highest identity (48%) to PSK-H1, a human kinase of unknown function (J. Woodgett, personal communication), rather than to other MLCKs (37–39% identical). It will be interesting to see whether PSK-HI functions as a calmodulin-independent MLCK. It will also be interesting to see whether the additional MLCK activities in *Dictyostelium* are Ca^{2+}/calmodulin-dependent, rho-activated, similar to MAPKAP kinase, or regulated in some other way. The availability of the MLCK-A$^-$ cells should facilitate characterization of the additional MLCKs.

MLCK-A and Cytokinesis. Our observation that MLCK-A$^-$ cells are multinucleate indicates that MLCK-A is needed for efficient cytokinesis, but the fact that the cells grow in suspension indicates that it is not essential for this process. The similar doubling times of wild-type and MLCK-A$^-$ cells is not surprising if one keeps in mind that a multinucleate cell can potentially undergo cytokinesis successfully after the next round of mitosis (49). We have observed a slight bias toward multinucleate cells with an even number of nuclei (Fig. 2). This may mean that in a multinucleate cell, if cytokinesis fails, it is most likely to fail completely. For example, at cytokinesis a tetranucleate cell is more likely to successfully divide into four mononucleate cells or remain tetranucleate than to end up as one trinucleate cell and one mononucleate cell.

One model to explain our results is that during cytokinesis MLCK-A is activated, resulting in phosphorylation of all the RLC in the cell, whereas a MLCK-A$^-$ cell must do the best it can with partially phosphorylated myosin. While it is not known whether RLC phosphorylation is induced during cytokinesis in *Dictyostelium* cells, in cultured mammalian cells a 20-fold increase in Ser-19 phosphorylation has been observed during cytokinesis (16). Thus it is simplest to consider a direct role for MLCK-A in cytokinesis—i.e., that MLCK-A is activated prior to furrowing. If this is the case, then understanding the regulation of MLCK-A should help elucidate the signaling pathways used during cytokinesis. The Con A-induced activation of MLCK-A provides an excellent system for studying its *in vivo* regulation, and we have recently exploited this system to show that MLCK–A is activated by phosphorylation by an upstream kinase (20). We have also shown that MLCK-A is activated in lysates by cGMP (manuscript in preparation). In Novikoff rat hepatoma cells, a peak of cGMP has been observed during mitosis (50). One intriguing possibility is that

cGMP and a cGMP-dependent protein kinase may involved in signaling during cytokinesis in *Dictyostelium*.

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1. De Lozanne, A. & Spudich, J. A. (1987) *Science* **236,** 1086–1091.
2. Knecht, D. & Loomis, W. F. (1987) *Science* **236,** 1081–1086.
3. Manstein, D. M., Titus, M. A., De Lozanne, A. & Spudich, J. (1989) *EMBO J.* **8,** 923–932.
4. Pollenz, R. S., Chen, T. L., Trivinos-Lagos, L. & Chisholm, R. L. (1992) *Cell* **69,** 951–962.
5. Chen, P., Ostrow, B. D., Tafuri, S. R. & Chisholm, R. L. (1994) *J. Cell Biol.* **127,** 1933–1944.
6. Chen, T. L., Kowalczyk, P. A., Ho, G. & Chisholm, R. L. (1995) *J. Cell. Sci.* **108,** 3207–3218.
7. Pasternak, C., Spudich, J. A. & Elson, E. L. (1989) *Nature (London)* **341,** 549–551.
8. Rappaport, R. (1986) *Int. Rev. Cytol.* **105,** 245–281.
9. Satterwhite, L. L. & Pollard, T. D. (1992) *Curr. Opin. Cell Biol.* **4,** 43–52.
10. Fishkind, D. J. & Wang, Y.-L. (1995) *Curr. Opin. Cell Biol.* **7,** 23–31.
11. Trybus, K. (1991) *Cell Motil. Cytoskel.* **18,** 55–81.
12. Sellers, J. (1991) *Curr. Opin. Cell Biol.* **3,** 98–104.
13. Tan, J. L., Ravid, S. & Spudich, J. A. (1992) *Annu. Rev. Biochem.* **61,** 721–759.
14. Sweeney, H. L., Bowman, B. F. & Stull, J. T. (1993) *Am. J. Physiol.* **264,** C1085–C1095.
15. Satterwhite, L. L., Lohka, M. J., Wilson, K. L., Scherson, T. Y., Cisek, L. J., Corden, J. L. & Pollard, T. D. (1992) *J. Cell Biol.* **118,** 595–605.
16. Yamakita, Y., Yamashiro, S. & Matsumura, F. (1994) *J. Cell Biol.* **124,** 129–137.
17. Griffith, L. M., Downs, S. M. & Spudich, J. A. (1987) *J. Cell Biol.* **104,** 1309–1323.
18. Tan, J. L. & Spudich, J. A. (1990) *J. Biol. Chem.* **265,** 13818–13824.
19. Tan, J. L. & Spudich, J. A. (1991) *J. Biol. Chem.* **266,** 16044–16049.
20. Smith, J. L., Silveira, L. A. & Spudich, J. A. (1996) *EMBO J.*, in press.
21. Sussman, M. (1987) *Methods Cell Biol.* **28,** 9–30.
22. Berlot, C. H., Spudich, J. A. & Devreotes, P. N. (1985) *Cell* **43,** 307–314.
23. Franke, J. & Kessin, R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 2157–2161.
24. Dynes, J. L. & Firtel, R. A. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 7966–7970.
25. Insall, R., Kuspa, A., Lilly, P. J., Shaulsky, G., Levin, L. R., Loomis, W. F. & Devreotes, P. (1994) *J. Cell. Biol.* **126,** 1537–1545.
26. Hadwiger, J. A. & Firtel, R. A. (1992) *Genes Dev.* **6,** 38–49.
27. Harlow, E. & Lane, D. (1988) *Antibodies: A Laboratory Manual*, (Cold Spring Harbor Lab. Press, Plainview, NY).
28. Rothblatt, J. & Schekman, R. (1989) *Methods Cell Biol.* **32,** 3–35.
29. Perrie, W. T. & Perry, S. V. (1970) *J. Biochem.* **119,** 31–38.
30. Peltz, G., Spudich, J. A. & Parham, P. (1985) *J. Cell Biol.* **100,** 1016–1023.
31. Patterson, B. & Spudich, J. A. (1995) *Genetics* **140,** 505–515.
32. Ostrow, B. D., Chen, P. & Chisholm, R. L. (1994) *J. Biol. Chem.* **127,** 1945–1955.
33. Trybus, K. M. (1989) *J. Cell Biol.* **109,** 2887–2894.
34. Berlot, C. H., Devreotes, P. N. & Spudich, J. A. (1987) *J. Biol. Chem.* **262,** 3918–3926.
35. Lin, S. S. & Levitàn, I. B. (1991) *Trends Neurosci.* **14,** 273–277.
36. Manjarrez-Hernandez, H. A., Baldwin, T. J., Aitken, A., Knutton, S. & Williams, P. H. (1992) *Lancet* **339,** 521–523.
37. Maruta, J., Baltes, W., Dieter, P., Marme, D. & Gerisch, G. (1983) *EMBO J.* **2,** 535–542.
38. Kuczmarski, E. R. (1986) *J. Muscle Res. Cell Motil.* **7,** 501–509.
39. Côté, G. P. & Bukiejko, U. (1987) *J. Biol. Chem.* **262,** 1065–1072.
40. Ravid, S. & Spudich, J. A. (1989) *J. Biol. Chem.* **264,** 15144–15150.
41. Kolman, M. F., Futey, L. M. & Egelhoff, T. T. (1996) *J. Cell Biol.* **132,** 101–109.
42. Abuelneel, K., Karchi, M. & Ravid, S. (1996) *J. Biol. Chem.* **271,** 977–984.
43. Noegel, A. A. & Luna, J. E. (1995) *Experientia* **51,** 1135–1143.
44. Stull, J. T., Nunnally, M. H. & Michnoff, C. H. (1986) in *The Enzymes: Control by Phosphorylation, Part A,* eds. Boyer, P. D. & Krebs, E. G. (Academic, Orlando), Vol. 17, pp. 113–166.
45. Shoemaker, M. O., Lau, W., Shattuck, R. L., Kwiatkowski, A. P., Matrisian, P. E., Guerra-Santos, L., Wilson, E., Lukas, T. J., Van Eldik, L. J. & Watterson, D. M. (1990) *J. Cell Biol.* **111,** 1107–1125.
46. Okagaki, T., Ishikawa, R. & Kohama, K. (1991) *Eur. J. Cell Biol.* **56,** 113–22.
47. Komatsu, S. & Hosoya, H. (1996) *Biochem. Biophys. Res. Commun.* **223,** 741–745.
48. Amano, M., Ito, M., Kimura, K., Fukata, Y., Chihara, K., Nakano, T., Matsuura, Y. & Kaibuchi, K. (1996) *J. Biol. Chem.* **271,** 20246–20249.
49. Kitanishi-Yumura, T. & Fukui, Y. (1989) *Cell Motil. Cytoskeleton* **12,** 78–89.
50. Zeilig, C. E. & Goldberg, N. D. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 1052–1056.