The EMBO Journal vol.15 no.22 pp.6075–6083, 1996

Activation of *Dictyostelium* myosin light chain kinase A by phosphorylation of Thr166

Janet L.Smith, Linda A.Silveira[1] and James A.Spudich[2]

Department of Biochemistry, Beckman Center, Stanford University Medical Center, Stanford, CA 94305-5307, USA

[1]Present address: Department of Biology, 1200 E. Colton Avenue, PO Box 3080, University of Redlands, Redlands, CA 92373-0999, USA

[2]Corresponding author

Phosphorylation of the regulatory light chain is an important mechanism for the activation of myosin in non-muscle cells. Unlike most myosin light chain kinases (MLCKs), MLCK-A from *Dictyostelium* is not activated by Ca^{2+}/calmodulin. Autophosphorylation increases activity, but only to a low level, suggesting that there is an additional activation mechanism. Here, we show that MLCK-A is autophosphorylated on Thr289, which is C-terminal to the catalytic domain. Phosphorylation of MLCK-A increases in response to concanavalin A (conA) treatment of cells, which was previously shown to activate MLCK-A. However, a mutant kinase with an alanine at position 289 (T289A) is also phosphorylated *in vivo*, indicating that there is an additional phosphorylated residue. Based on comparisons with other protein kinases, we tested whether phosphorylation of Thr166 drives activation of MLCK-A. Our data indicate that phosphorylation of Thr289 occurs *in vivo*, but is not associated with conA-induced activation, whereas phosphorylation of Thr166 by some as yet unidentified kinase is associated with activation. Replacement of Thr166 with glutamate results in a 12-fold increase in activity as compared with the wild-type enzyme, supporting the idea that phosphorylation of Thr166 increases MLCK-A activity.

Keywords: concanavalin A/cytoskeleton/MLCK/phosphorylation/protein kinase

Introduction

While myosin II was originally studied as the molecular motor involved in muscle contraction, it is also found in non-muscle cells, where it is involved in a variety of cellular functions. *Dictyostelium discoideum* engineered to lack functional myosin are unable to undergo cytokinesis in shaking cultures (De Lozanne and Spudich, 1987; Knecht and Loomis, 1987; Manstein *et al.*, 1989; Pollenz *et al.*, 1992; Chen *et al.*, 1994, 1995) and those lacking the myosin heavy chain or the essential light chain are unable to cap concanavalin A (conA)-crosslinked surface proteins (Pasternak *et al.*, 1989; Pollenz *et al.*, 1992; Chen *et al.*, 1995). These cells are able to locomote toward a chemotactic signal, but do so less efficiently than wild-type cells (Wessels *et al.*, 1988). They are able to form aggregates during starvation, but the aggregates do not develop into fruiting bodies. While the role of myosin as a molecular motor during cytokinesis, fruiting body formation, and conA capping is well established, the signal transduction pathways regulating its activity during these processes are less well understood.

The actin-activated ATPase of myosin from muscle and non-muscle cells is increased by phosphorylation of the regulatory light chain (RLC) by myosin light chain kinase (MLCK). *In vitro*, RLC phosphorylation of *Dictyostelium* myosin has been shown to result in a 6-fold increase in steady-state ATPase activity. Furthermore, in an *in vitro* motility assay, 0.9% of beads coated with unphosphorylated myosin move on actin cables, whereas 75% of beads coated with phosphorylated myosin move (Griffith *et al.*, 1987). In addition, RLC phosphorylation is regulated during chemotaxis (Berlot *et al.*, 1985, 1987), and conA capping (Smith *et al.*, 1996). These findings suggest that RLC phosphorylation is an important regulatory mechanism during myosin-dependent cellular functions in *Dictyostelium*.

Dictyostelium cells have at least two MLCKs (Smith *et al.*, 1996). One of these (MLCK-A) has been biochemically characterized (Griffith *et al.*, 1987; Tan and Spudich, 1990) and the gene encoding it has been cloned (Tan and Spudich, 1991). This kinase differs rather markedly from most other MLCKs. Its molecular weight is 34 kDa, with a compact kinase domain followed by 39 amino acids at the C-terminus. The sequence of MLCK-A suggests that it would not bind or be activated by Ca^{2+}/calmodulin (Tan and Spudich, 1991), and this has been confirmed experimentally (Tan and Spudich, 1990). In contrast, most MLCKs are much larger, and require Ca^{2+}/calmodulin for activity (reviewed in Stull *et al.*, 1986). MLCK-A undergoes intramolecular autophosphorylation, which increases its activity (Tan and Spudich, 1990). However, the activity of fully autophosphorylated MLCK-A is still much lower than other MLCKs.

Dictyostelium cells in which the MLCK-A gene is disrupted are still able to complete multicellular development (Smith *et al.*, 1996). These cells grow in suspension, but the cultures have an increased frequency of multinucleate cells, indicating that MLCK-A is needed for efficient cytokinesis. The weak phenotype of these cells compared with cells which lack myosin may reflect the presence of residual phosphorylated RLCs. Alternatively, it is possible that RLC phosphorylation is not essential for all of myosin's functions *in vivo*. In a related study, cells which have a mutant RLC with an alanine at Ser13, the phosphorylation site, were found to be wild-type in all regards, including cytokinesis (Ostrow *et al.*, 1994). This surprising finding suggests that RLC phosphorylation

is not important for myosin function *in vivo*, and is difficult to reconcile with the effect of the MLCK-A gene disruption on cytokinesis, the activation of myosin by RLC phosphorylation observed *in vitro*, and the fact that RLC phosphorylation has been shown to be regulated during chemotaxis and conA capping. Regardless of how essential RLC phosphorylation may be to *Dictyostelium* cells, the fact that it occurs in such a well-regulated fashion suggests that it almost certainly provides some benefit to this organism. In addition, RLC phosphorylation provides a useful inroad for the elucidation of the signal transduction pathways regulating myosin-dependent processes such as conA capping, development and cytokinesis.

A number of observations point to an additional mechanism beyond autophosphorylation for the activation of MLCK-A. First of all, autophosphorylation alone would not allow MLCK-A to respond to external stimuli, yet it has been shown that MLCK-A is activated in response to conA *in vivo*, and in response to cGMP in lysates (L.A.Silveira, J.L.Smith and J.A.Spudich, manuscript in preparation). Also, MLCK-A is responsible for rapidly and quantitatively phosphorylating the RLC in response to conA (Smith *et al.*, 1996), yet the activity measured *in vitro* for fully autophosphorylated MLCK-A is too low to achieve this. In fact, we have estimated that the activity of MLCK-A in lysates from conA-treated cells is ~100-fold higher than that of purified, fully autophosphorylated MLCK-A (L.A.Silveira, J.L.Smith and J.A.Spudich, manuscript in preparation). Finally, a mutant MLCK-A which is unable to undergo autophosphorylation because it has an alanine at the autophosphorylation site is also activated in response to conA (L.A.Silveira, J.L.Smith and J.A.Spudich, manuscript in preparation).

Since MLCK-A appears to play a role in regulating myosin during cytokinesis and conA capping, we were interested in defining the upstream signals which regulate its activity, as a way of elucidating the signaling mechanisms used during these cellular functions. A variety of kinases are activated by phosphorylation, most commonly in the 'activation loop' between the conserved sequences DFG and APE (subdomains VII and VIII in the nomenclature of Hanks and Hunter, 1995). cAMP-dependent protein kinase (PKA) is activated by phosphorylation of Thr197 in this region (Shoji *et al.*, 1983; Steinberg *et al.*, 1993). The crystal structure of Thr197-phosphorylated PKA revealed that the phosphate group interacts with several basic residues throughout the catalytic domain (Knighton *et al.*, 1991a,b, 1993; Bossemeyer *et al.*, 1993; Zheng *et al.*, 1993). Structures have also been determined for cdk2 (De Bondt *et al.*, 1993), erk2 (Zhang *et al.*, 1994), the insulin receptor (Hubbard *et al.*, 1994) and calcium/calmodulin-dependent protein kinase I (Goldberg *et al.*, 1996), each of which requires phosphorylation in the activation loop, but which were crystallized in the unphosphorylated form. In addition, structures of twitchin (Hu *et al.*, 1994), casein kinase 1 (Xu *et al.*, 1995) and phosphorylase kinase (Owen *et al.*, 1995), which do not undergo activation loop phosphorylation, have been solved. From these various studies, an understanding of the structural basis for regulation by phosphorylation in the activation loop is beginning to emerge (reviewed in Cox *et al.*, 1994; Goldsmith and Cobb, 1994; Morgan and De Bondt, 1994; Johnson *et al.*, 1996).

Table I. Sequence of the ^{32}P-labeled peptide from autophosphorylated, endoproteinase AspN-digested MLCK-A[a]

Amino acid predicted from DNA sequence	Amino acid detected	Yield (pmol)
275-D	D	1080
T	T	310
V	V	1050
K	K	1015
M	M	1050
280-K	K	1020
E	E	507
Y	Y	501
I	I	415
V	V	445
285-E	E	353
R	R	341
Q	Q	257
K	K	228
289-T	–	–
290-Q	Q	34
T	T	20
K	K	26
L	L	16
V	V	17
295-N	(N)	4
STOP	–	–

[a]The yield from each cycle on the automated sequencer is given. In the course of this work, a frameshift error in the sequence of this region of MLCK-A was discovered. The correct sequence has been submitted to GenBank.

We have characterized the *in vivo* phosphorylation of MLCK-A, to examine whether it is activated by phosphorylation. We found that phosphorylation of MLCK-A increases in response to conA treatment. We determined that autophosphorylation occurs on Thr289, and that although phosphorylation of this residue occurs *in vivo*, it does not increase substantially in response to conA. Phosphorylation of a second site—Thr166—is associated with activation by conA. By comparison with other protein kinases, phosphorylation of this residue is predicted to be activating. To test this we mimicked phosphorylation by mutating this residue to a glutamate. T166E MLCK-A had 12-fold higher enzyme activity *in vitro*, reinforcing the conclusion that MLCK-A is activated *in vivo* by Thr166 phosphorylation.

Results

MLCK-A is autophosphorylated at Thr289

HPLC separation of an endoproteinase Asp-N digest of MLCK-A resulted in one ^{32}P-labeled peptide peak. N-terminal sequencing of this peptide revealed that it was the most C-terminal fragment of MLCK-A, beginning with residue 275 (Table I). At position 289, a threonine in the MLCK-A sequence, a blank cycle was obtained, which is consistent with a phosphothreonine at this position. After this position, yields decreased considerably, and a high amount of lag in the sequence was observed. Taking the lag into account, an unambiguous sequence matching residues 290–295 of the predicted MLCK-A sequence was obtained. The decreased threonine yield at position 276 is due to the fact that Edman degradation results in three products for threonine residues, only one of which is routinely quantified. However, there was

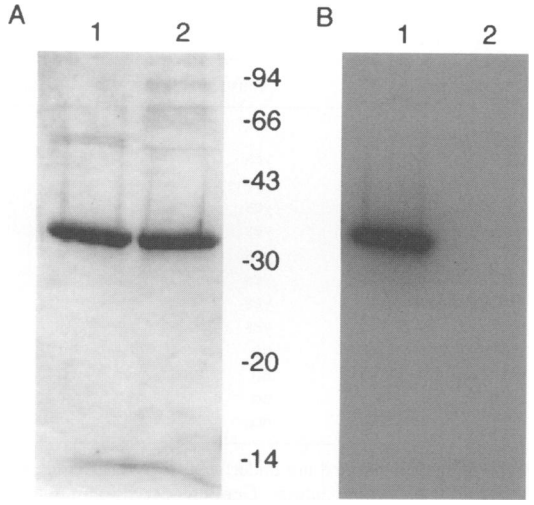

Fig. 1. Autophosphorylation of wild-type and T289A MLCK-A. Recombinant wild-type or T289A MLCK-A was allowed to undergo autophosphorylation with [γ-^{32}P]ATP and then electrophoresed on a 15% SDS–polyacrylamide gel. (**A**) Coomassie stain. (**B**) Autoradiograph of the same gel. Lane 1, 3 μg wild-type MLCK-A; lane 2, 3 μg T289A MLCK-A. The molecular weight standards shown are in kDa.

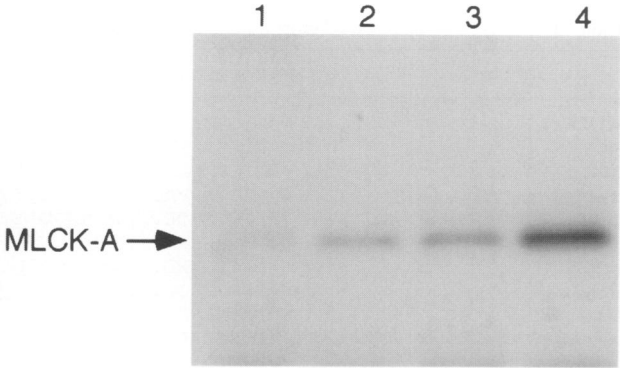

Fig. 2. Phosphate incorporation into wild-type and T289A MLCK-A *in vivo*. Endogenous wild-type MLCK-A (lanes 1 and 2) or T289A MLCK-A expressed from a plasmid in MLCK-A$^-$ cells (lanes 3 and 4) was immunoprecipitated from ^{32}P-labeled cells which were either untreated (lanes 1 and 3) or conA-treated (lanes 2 and 4) The immunoprecipitates were electrophoresed on a 15% SDS–polyacrylamide gel, and the gel was exposed to film. The gel was also exposed to a PhosphorImager screen and ^{32}P incorporation into MLCK-A was quantified using ImageQuant v. 3.22 software (Molecular Dynamics, Sunnyvale, CA). The higher level of labeling on T289A MLCK-A is due to the fact that it is ~10-fold overexpressed compared with the endogenous wild-type MLCK-A.

sufficient signal for Lys288 that Thr289 would have been readily identified had it been PTH-threonine.

To confirm that Thr289 is the autophosphorylation site, this position was mutated to an alanine residue (T289A). Lysates from *Escherichia coli* expressing either wild-type or T289A MLCK-A were assayed for autophosphorylation by incubating them with [γ-^{32}P]ATP. High levels of ^{32}P were incorporated into wild-type MLCK-A, and none was incorporated into T289A (Figure 1).

MLCK-A contains phosphothreonine in vivo

Since MLCK-A undergoes autophosphorylation of Thr289 *in vitro*, it seemed likely that MLCK-A would be phosphorylated on this position, and possibly additional positions, *in vivo*. To see if MLCK-A is a phosphoprotein *in vivo*, MLCK-A was immunoprecipitated from a wild-type strain (JH10, Hadwiger and Firtel, 1992) which had been metabolically labeled with ^{32}P. ^{32}P-labeled MLCK-A was recovered (Figure 2, lane 1) and phosphoamino acid analysis revealed that all of the ^{32}P incorporation was on threonine (Figure 3).

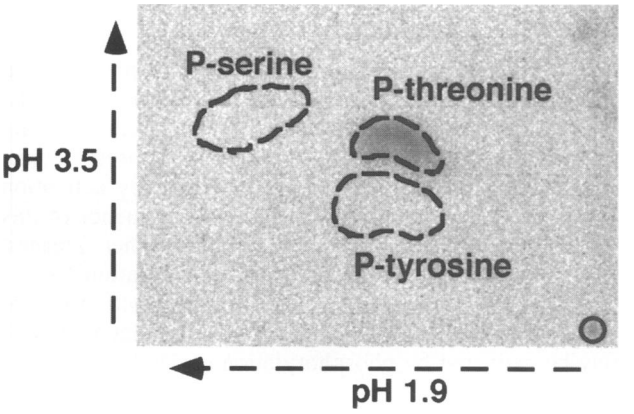

Fig. 3. Phosphoamino acid analysis of *in vivo* labeled MLCK-A. MLCK-A was immunoprecipitated from ^{32}P-labeled cells, hydrolyzed to amino acids, and subjected to two-dimensional thin-layer electrophoresis. P-serine, P-threonine and P-tyrosine standards were detected by ninhydrin staining, and the plate was exposed to a PhosphorImager screen to detect ^{32}P-labeled amino acids from MLCK-A.

ConA increases the phosphorylation of wild-type and T289A MLCK-A in vivo

Since conA treatment of cells activates MLCK-A (Smith *et al.*, 1996), we investigated whether conA treatment results in a change in MLCK-A phosphorylation. The level of ^{32}P incorporation in MLCK-A immunoprecipitated from labeled JH10 cells was 2- to 3-fold higher in conA-treated cells than in untreated cells (Figure 2, lanes 1 and 2). To see if this was due to autophosphorylation, we expressed T289A MLCK-A in MLCK-A$^-$ *Dictyostelium* cells using the promoter for the *Dictyostelium* actin15 gene (Cohen *et al.*, 1986). This expression system resulted in ~10-fold overexpression of MLCK-A, as estimated by immunoblotting (data not shown). ^{32}P-labeled T289A kinase was immunoprecipitated from these cells, and the level of phosphorylation increased 6- to 7-fold upon conA treatment (Figure 2, lanes 3 and 4).

The sequence of MLCK-A suggests that it may be regulated by phosphorylation in the activation loop

A number of protein kinases are activated by phosphorylation of residues in the activation loop, which is positioned at the mouth of the active site (for reviews, see Goldsmith and Cobb, 1994; Morgan and De Bondt, 1994; Johnson *et al.*, 1996). In PKA, the catalytic domain of which is 35% identical to MLCK-A, Thr197 is the site of this activating phosphorylation (Shoji *et al.*, 1983; Steinberg *et al.*, 1993), and the crystal structure of PKA revealed that this phosphothreonine makes contacts with three basic amino acids dispersed throughout the primary sequence (Knighton *et al.*, 1991a,b, 1993; Bossemeyer *et al.*, 1993;

Table II. Sequence comparison of protein kinases in the connector between subdomains VII and VIII[a]

	Subdomain VII–VIII connector (activation loop)	Activated by phosphorylation?
MLCK-A	^{151}DFGLS**K**II....GQTLVMQTACGTPSYVAPE^{177}	?
PKA	^{185}DFGFA**K**RV......KGRTW**T**LCGTPEYLAPE^{209}	yes
cdk2	^{145}DFGLA**R**AF...GVPVRTY**T**HEVVTLWYRAPE^{172}	yes
erk2	^{165}DFGLA**R**VADPDHDHTGTL**T**E**Y**VATRWYRAPE^{195}	yes
mek1	^{208}DFGVSGQL.....ID**S**MAN**S**FVGTRSYMSPE^{233}	yes
PKC	^{481}DFGMC**K**EH...MMDGVTTRTFCGTPDYIAPE^{508}	yes
CaMK1	^{162}DFGLS**K**ME. ..DPGSVLS**T**ACGTPGYVAPE^{183}	yes
GSK3	^{262}DFGSA**K**QL....VRGEPNVS**Y**ICSRYYRAPE^{288}	yes
InsR	^{1138}DFGMT**R**DI.**Y**ETD**YY**RKGGKGLLPVRWMAPE^{1167}	yes
twn	^{6083}DFGLT**A**HL....DPKQSVKVTTGTAEFAAPE^{6109}	no
CaMK2	^{156}DFGLAIEV...EGEQQAWFGFAGTPGYLSPE^{183}	no
CKII	^{175}DWGLAEFY....HPGQEYNVRVASRYFKGPE^{201}	no
PhK	^{167}DFGFSCQL....DPGEKLREVCGTPSYLAPE^{193}	no

[a]Gaps are indicated by dots. Threonine, serine and tyrosine residues which are known to be phosphorylated are underlined. In kinases containing a lysine or arginine at the position homologous to Lys189 of PKA, this residue is double-underlined. Abbreviations, GenBank accession numbers and references for activation loop phosphorylation are as follows: MLCK-A, *D.discoideum* myosin light chain kinase A (#M64176); PKA, human cAMP-dependent protein kinase Cα subunit (#X07767; Steinberg *et al.*, 1993); cdk2, human cyclin-dependent kinase 2 (#X61622, Ducommun *et al.*, 1991; Solomon *et al.*, 1992); erk2, rat extracellular signal-regulated kinase 2 (#M64300, Ahn *et al.*, 1991; Payne *et al.*, 1991; Robbins *et al.*, 1993); CaMK1, Calcium/calmodulin-dependent protein kinase I (#L26288; Mochizuki *et al.*, 1993; Picciotto *et al.*, 1993; Lee and Edelman, 1995); mek1, mouse MAPK/Erk kinase (#L02526, Huang and Erickson, 1994; Yan and Templeton, 1994); PKC, rat protein kinase Cα subunit (#X07286; Orr and Newton, 1994); GSK3, rat glycogen synthase kinase-3α (#X53427, Hughes *et al.*, 1993); InsR, human insulin receptor, precursor A (#X02160, White *et al.*, 1988); Twn, *Caenorhabditis elegans* twitchin (#L10351); CaMK2, rat Ca^{2+}/calmodulin-dependent protein kinase II α subunit (#J02942); CKII, human casein kinase II α subunit (#J02853); PhK, rabbit phosphorylase kinase γ_m subunit (#Y00684).

Zheng *et al.*, 1993). One of these residues—His87—is not conserved at all, even among other kinases which undergo phosphorylation in the activation loop. The second—Arg165—is almost universally conserved among protein kinases. The third—Lys189—is conserved predominantly in kinases which are regulated by activation loop phosphorylation. Table II shows the sequence of this region for MLCK-A and a variety of other kinases. Since MLCK-A has a threonine in the activation loop—Thr166—and a lysine at residue 156, the position homologous to Lys189 of PKA, we hypothesized that MLCK-A may be activated by phosphorylation of Thr166.

In vivo phosphorylation of T166A and T166A/T289A MLCK-A

In order to assess the possible *in vivo* role of Thr166 phosphorylation in the activation of MLCK-A, two mutant MLCK-As, T166A and T166A/T289A, were overexpressed in MLCK-A$^-$ *Dictyostelium*. ^{32}P-labeled T166A MLCK-A was immunoprecipitated from metabolically labeled cells, and the level of ^{32}P incorporation increased slightly (1.3-fold) in response to conA treatment (Figure 4). Since the ^{32}P incorporation in T166A MLCK-A was likely to be due to the autophosphorylation site, we also examined the double mutant T166A/T289A. No ^{32}P incorporation into this mutant MLCK-A was observed.

T166E MLCK-A is a 12-fold more active enzyme than wild-type

In order to test whether phosphorylation of Thr166 would result in the activation of MLCK-A, we partially mimicked phosphorylation by mutating this residue to a glutamate. Both wild-type and T166E MLCK-A were expressed in *E.coli* with a histidine tag on the N-terminus. The expression level of the T166E mutant was consistently approximately two-thirds that of wild-type (Figure 5, lanes 1 and 3), and the kinases were expressed in a soluble form (Figure 5, lanes 2 and 4). The T166E mutant migrated more slowly on SDS–polyacrylamide gels; this difference is more dramatic on higher percentage gels. The proteins, with the histidine tag removed by proteolysis with thrombin, were purified to near-homogeneity (Figure 5). The minor band at ~30 kDa results from thrombin cleavage of the MLCK-A at a secondary site. The amount of this product varied depending on the cleavage conditions used, but the activities of the preparations were comparable, suggesting that this product has activity similar to or less than that of the full-length MLCK-A. The k_{cat}/K_m value for wild-type MLCK-A purified in this manner was 670 ± 40 M^{-1} s^{-1} (Figure 6), which is similar to that reported by Tan and Spudich (1990). The activity of T166E MLCK-A was 12-fold higher (k_{cat}/K_m = 7900 ± 200 M^{-1} s^{-1}). We were not able to attain myosin concentrations in our assays that were high enough for us to determine K_m and k_{cat}.

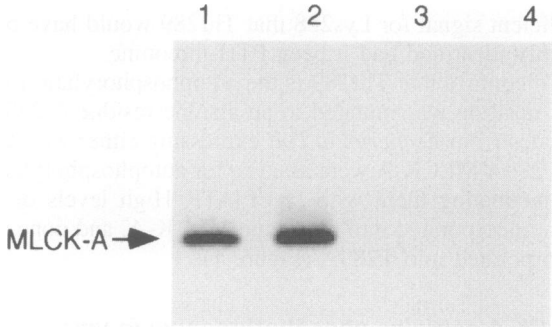

Fig. 4. Phosphate incorporation into T166A and T166A/T289A MLCK-A *in vivo*. T166A MLCK-A (lanes 1 and 2) or T166A/T289A (lanes 3 and 4) MLCK-A expressed from a plasmid was immunprecipitated from ^{32}P-labeled cells which were either untreated (lanes 1 and 3) or conA treated (lanes 2 and 4). The immunoprecipitates were electrophoresed on a 15% SDS–polyacrylamide gel, and the gel was exposed to a PhosphorImager screen. ^{32}P incorporation into MLCK-A was quantified using ImageQuant v.3.22 software (Molecular Dynamics, Sunnyvale, CA).

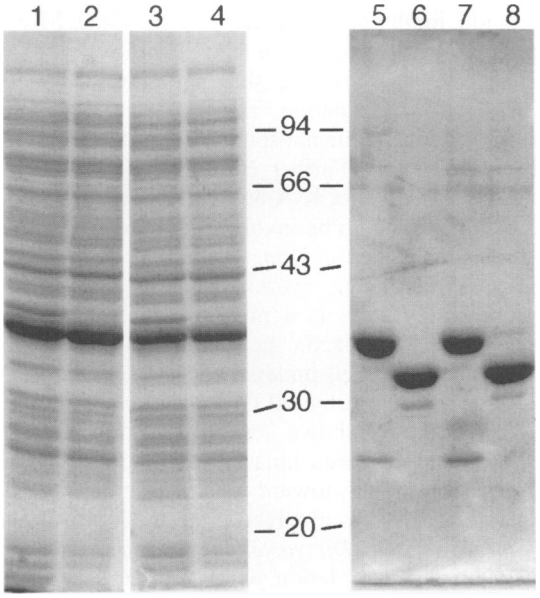

Fig. 5. Purification of wild-type and T166E MLCK-A using a His_6 tag. Coomassie-stained 12% polyacrylamide gel of samples from the purification of wild-type and T166E MLCK-A using a $(His)_6$ tag. Lane 1, whole cell lysate; lane 2, soluble fraction, from *E.coli* expressing His-tagged wild-type MLCK-A; lane 3, whole-cell lysate; lane 4, soluble fraction, from *E.coli* expressing His-tagged T166E MLCK-A; lane 5, 5 μg purified His-tagged wild-type MLCK-A; lane 6, 5 μg wild-type MLCK-A after thrombin cleavage and reincubation with the Ni^{2+} resin; lane 7, 5 μg purified His-tagged T166E MLCK-A; lane 8, 5 μg T166E MLCK-A after thrombin cleavage and reincubation with the Ni^{2+} resin.

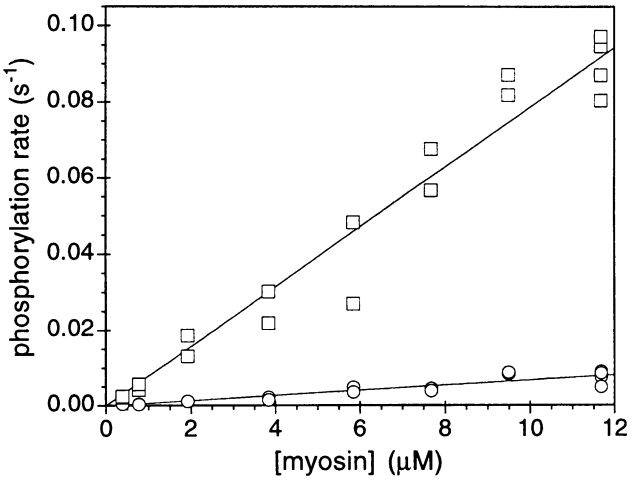

Fig. 6. Activity of recombinant wild-type and T166E MLCK-A. MLCK-A activity was measured using as a substrate a mutant form of *Dictyostelium* myosin which does not hydrolyze ATP. The molecular weight of *Dictyostelium* myosin is 274 kDa, including the two light chains. ○, wild-type MLCK-A; □, T166E MLCK-A. Both wild-type and T166E MLCK-A were fully autophosphorylated, and the wild-type was unphosphorylated on Thr166. The data are fitted to a straight line, the slope of which is k_{cat}/K_m.

Discussion

Identification of the phosphorylation sites on MLCK-A

It was predicted from previous studies with a truncated form of the kinase that MLCK-A would be autophosphorylated on a threonine in the last 39 residues of the protein—outside of the kinase domain (Tan and Spudich, 1991). We used peptide sequencing to show that the autophosphorylated residue is Thr289, which, as predicted, is C-terminal to the kinase domain. A mutant kinase with an alanine at this position does not undergo autophosphorylation, which further strengthens the assignment of Thr289 as the autophosphorylation site.

Since MLCK-A has very high protein substrate specificity (Tan and Spudich, 1990), one might expect that there would be strong homology between the phosphorylation site on the RLC (Tafuri *et al.*, 1989; Ostrow *et al.*, 1994) and the autophosphorylation site. This, however, is not the case. The only conserved residues between the two substrates is an arginine that is three residues before the phosphoacceptor position (P–3), and a leucine that is four residues after the phosphoacceptor position (P+4). For other MLCKs, a constellation of basic residues N-terminal to the phosphoacceptor position, and hydrophobic residues two and three residues C-terminal to the phosphorylation site, are important for substrate recognition (see Zhi *et al.*, 1994 and references therein). While the arginine at the P–3 position almost certainly makes important contacts with the catalytic core of MLCK-A, the significance of the conserved leucine at P+4 is unclear. The sequence surrounding the Thr289 site suggests that it is a poor substrate, although the fact that autophosphorylation is intramolecular probably enhances the reaction somewhat. However, it would be detrimental for this region to bind too tightly, because it might continue to bind to the active site after autophosphorylation has occurred and continue to inhibit the enzyme. Given the slow rate of autophosphorylation ($t_{1/2}$ = 22 min; Tan and Spudich, 1990), it is probably worth keeping in mind the possibility that phosphorylation of Thr289 is actually carried out *in vivo* by a different kinase.

The second *in vivo* phosphorylation site—Thr166—was identified quite differently. Rather than directly characterizing the phosphoprotein, we made a guess that this residue is phosphorylated, and tested the guess by mutating Thr166 to an alanine. The fact that T166A/T289A MLCK-A is not phosphorylated *in vivo* strongly suggest that these are the only two phosphorylation sites, although it is also possible that the mutations inhibit phosphorylation of an additional site(s).

Thr166 lies within the activation loop, and Yonemoto *et al.* (1993) have suggested that many kinases which are inactive when expressed in *E.coli* may require phosphorylation in this region for activity. Indeed, a variety of protein kinases have been found to be activated by phosphorylation in this region. The crystal structure of PKA revealed that the phosphothreonine at location 197 on this loop interacts with Lys189, and it appears that the presence of a phosphorylatable residue in the C-terminal part of the loop coupled with a lysine or arginine at position 189 correlates well with regulation by activation loop phosphorylation (Table II). One exception is mek1, which undergoes activation loop phosphorylation, yet has a glycine at this position (Huang and Erickson, 1994; Yan and Templeton, 1994).

How is MLCK-A regulated?

Since phosphorylation of wild-type MLCK-A increases in response to conA treatment, it seems likely that MLCK-A

is activated by phosphorylation. But phosphorylation of which residue, Thr166 or Thr289, is responsible for activation? Some insight into this question can be gained by looking at the response to conA of T166A MLCK-A and T289A MLCK-A. When phosphorylation of Thr289 is eliminated by mutating this position to an alanine, the increase in phosphorylation of MLCK-A in response to conA is much more dramatic than that observed for the wild-type enzyme. This is consistent with a scenario where Thr289 is constitutively phosphorylated, whereas Thr166 is phosphorylated in response to conA. If this is the case, then we would predict that phosphate incorporation into wild-type MLCK-A should double with conA treatment, if the entire pool of kinase is phosphorylated. This is approximately what we observed. This model also predicts that the phosphorylation of T166A MLCK-A should not change much with conA treatment, which was also borne out experimentally.

An important point in this model is that phosphorylation of Thr166 increases MLCK-A activity. Although sequence comparisons with other protein kinases such as PKA support the conclusion that Thr166 phosphorylation would be activating, ideally this question should be addressed experimentally by assaying MLCK-A which is phosphorylated at this position. However, MLCK-A phosphorylated at Thr166 was not readily available to us, because MLCK-A does not undergo autophosphorylation at this site, and we have not yet isolated the upstream kinase. We avoided this problem by mutating this residue to a glutamate in hopes of partially mimicking the effects of phosphorylation. In other systems, it has been found that the negative charge and overall size and shape of a glutamate or aspartate residue sufficiently resembles that of a phosphothreonine that the mutation partially reproduces the effect of phosphorylation. This approach was introduced using isocitrate dehydrogenase (Thorsness and Koshland, 1987), and has been used successfully in other systems (Egelhoff *et al.*, 1993; Sweeney *et al.*, 1994) including activation loop phosphorylation in protein kinases (Ducommun *et al.*, 1991; Gould *et al.*, 1991; Robbins *et al.*, 1993; Huang and Erickson, 1994; Orr and Newton, 1994; Yan and Templeton, 1994), although careful characterization of the mutant proteins often reveals that the acidic residues only partially reproduce the effects of phosphorylation.

We found that the T166E mutation increased the activity of MLCK-A 12-fold. We have estimated that MLCK-A in lysates from conA-stimulated cells is ~100-fold more active than purified recombinant enzyme (L.A.Silveira, J.L.Smith and J.A.Spudich, manuscript in preparation). Since a glutamate only approximates a phosphothreonine, and since this residue is predicted to make several specific interactions with other residues in the kinase, rather than simply acting as a repulsive negative charge, the 12-fold increase in activity compares quite favorably with the predicted 100-fold increase in activity from actual phosphorylation at this position.

If phosphorylation of Thr166 is the main switch to turn on MLCK-A, then what is the role of the C-terminal tail of MLCK-A, and of autophosphorylation? Our data indicate that phosphorylation of Thr289 is not required for phosphorylation at Thr166, since T289A MLCK-A is phosphorylated in response to conA. However, autophosphorylation might be involved in controlling MLCK-A by affecting the binding of the Thr166 phosphatase. Alternatively, it is possible that autophosphorylation is not involved in regulation, i.e. it occurs as a protein maturation step, and the phosphate at Thr289 then remains stably incorporated. Further experiments are needed to clarify the role of MLCK-A autophosphorylation.

Myosin is known to be involved in a variety of cellular processes in *Dictyostelium*, including conA-induced capping, development, cytokinesis and cell migration. While its importance as a motor in these processes is quite well characterized, the pathways underlying its regulation are less well understood, and of keen interest. RLC phosphorylation by MLCK-A activates the myosin motor, and we have shown here that MLCK-A in turn is activated by an upstream kinase. This finding thus represents a further step toward characterizing the signal transduction pathways involved in regulation of the actomyosin cytoskeleton. *Dictyostelium* myosin also undergoes heavy chain phosphorylation, which regulates its assembly into filaments (reviewed in Tan *et al.*, 1992; Hammer, 1994). Heavy and light chain phosphorylation of myosin, together with the wide variety of mechanisms regulating actin (reviewed in Noegel and Luna, 1995), undoubtedly provide a rich system for the temporal and spatial regulation of the actomyosin cytoskeleton.

Materials and methods

Expression of wild-type, mutant and $(His)_6$-tagged MLCK-As in E.coli

The T7 polymerase-driven expression plasmid pET15b (Novagen, Madison, WI) was used for the expression of MLCK-As tagged at the N-terminus with $(His)_6$. This vector allows the $(His)_6$ tag to be removed by cleavage with thrombin, leaving the three residues Gly-Ser-His from the vector at the N-terminus. For all MLCK-A mutants, a cDNA-derived clone of MLCK-A in mp19 was modified by site-directed mutagenesis as described in Kunkel (1987). An *Nde*I site was placed at the start ATG codon using the antisense mutagenic oligonucleotide 5′-cttctgc**catatg**atatctccttc-3′, where the *Nde*I site is in bold, and mismatches with the template are underlined. For generating the T166E mutation, a second antisense mutagenic primer 5′-tgtaccacatgcttcttgcattacaag-3′ was also included in the mutagenesis reaction. The mutant clones were identified by DNA sequencing. The 0.9 kb *Nde*I–*Bam*HI fragment containing the entire coding sequence of the MLCK-A cDNA from these modified clones was inserted into *Nde*I–*Bam*HI-digested pET15b. The resulting expression plasmids were transformed into the *E.coli* strain BL21 (DE3) pLysS.

Untagged MLCK-A was also expressed in *E.coli* using the T7 polymerase system. A 0.9 kb *Nco*I–*Bam*HI fragment was purified from a plasmid containing a full-length MLCK-A cDNA with an *Nco*I site engineered at the start ATG (Tan and Spudich, 1991) and a *Bam*HI site 3′ of the open reading frame. The *Nco*I site changes the second codon from a threonine to an alanine. This fragment was inserted into *Nco*I–*Bam*HI-digested pET11d (Novagen, Madison, WI). The T289A mutation was generated using the antisense oligonucleotide 5′-ttttgtttgagctttt-tgtctttc-3′. The 0.9 kb *Nco*I–*Bam*HI fragment containing this mutation was also inserted into pET11d to generate a plasmid for the expression in *E.coli* of untagged T289A MLCK-A.

Purification of His-tagged MLCK-A from E.coli

Cells were grown in 0.5 l of LB medium containing 34 μg/ml chloramphenicol and 100 μg/ml carbanicillin at 28–29°C. (At ≥30°C, MLCK-A is expressed in an insoluble form.) When the A_{600} reached 0.3–0.6, the culture was induced with 0.4 mM isopropyl β-D-thiogalactopyranoside (IPTG). The cells were harvested 3–5 h later at 7000 r.p.m. (8700 *g*) in a Beckman (Columbia, MD) JA10 rotor for 7 min. The supernatant was removed completely, and the cell pellet was resuspended in 10 ml binding buffer (20 mM Tris–HCl, pH 7.9, 5 mM imidazole, 500 mM NaCl) and frozen at –70°C. The cells lysed upon thawing. $MgCl_2$ was

added to 10 mM, phenylmethylsulfonyl fluoride (PMSF) to 1 mM, and DNase I (bovine pancreas grade II; Boehringer Mannheim, Indianapolis, IN) to 5 μg/ml. The lysate was stirred at 22°C until the viscosity decreased (~15 min), then spun for 15 min at 4°C in a Beckman JA20 rotor at 17 000 r.p.m. (35 000 *g*). The supernatant was added to 1 ml Novagen (Madison, WI) nickel resin prepared according to the manufacturer's instructions, and rotated at 4°C for 30 min. The resin was transferred to a column and washed with 10 ml binding buffer followed by 10 ml wash buffer (same as binding buffer but with 25 mM imidazole). About half of the kinase was lost in this wash. The bound kinase was eluted with elution buffer (same as binding buffer, except 200 mM imidazole). The fractions containing MLCK-A were dialyzed against thrombin cleavage buffer (20 mM Tris–HCl, pH 8.4, 150 mM NaCl, 2.5 mM $CaCl_2$). 0.2–0.5 units thrombin (Novagen, Madison, WI) was added per mg kinase, and the digest was allowed to proceed at 22°C for 1.5–3 h, and then stopped by the addition of PMSF to 1 mM. The digest was rotated at 4°C for 30 min with 1 ml prepared nickel resin equilibrated with binding buffer. The mixture was transferred to a column, and the flow-through was stored on ice. MLCK-A activity remained constant for at least 1 week. For wild-type MLCK-A, ~14 mg of protein were recovered, and for T166E, 9 mg were recovered.

Purification of untagged MLCK-A from E.coli

Cells from 3 l LB media were grown, induced and harvested as described above for the purification of His-tagged MLCK-A. The supernatant was removed completely and the cell pellet resuspended in 100 ml buffer A [50 mM Tris–HCl, 50 mM KCl, 5 mM ethylenediaminetetra-acetic acid (EDTA), 1 mM PMSF, 1 mM dithiothreitol (DTT), pH 7.5] and frozen at –70°C. The thawed cells were sonicated on ice with stirring using a Model 450 sonifier (Branson Ultrasonics, Danbury, CT) fitted with a pre-chilled 1 cm probe. The output was set at 5.5, the duty cycle was set at 50%, and the mixture was sonicated twice for 1 min, with at least 2 min for cooling in between. The lysate was spun for 15 min at 17 000 r.p.m. (35 000 *g*) in a Beckman JA20 rotor. Protamine sulfate was added to the supernatant from a 1% stock to a final concentration of 0.13%. The mixture was stirred for 10 min at 4°C, and then spun for 15 min at 10 000 r.p.m. (15 000 *g*) in a JA14 rotor. A 70–80% ammonium sulfate cut was prepared as follows. The supernatant was made 50% saturated with solid ammonium sulfate, and after stirring for 15 min was centrifuged for 10 min at 10 000 r.p.m. (15 000 *g*) in a JA14 rotor. The supernatant was made 70% saturated with solid ammonium sulfate and centrifuged as described above. The saturation of ammonium sulfate in the supernatant was increased to 80% and the precipitate was recovered by centrifugation as described above. The 70–80% ammonium sulfate pellet was dissolved in 10 ml buffer B (same as buffer A except 0.5 M ammonium sulfate was added), and clarified by centrifugation at 15 000 r.p.m. (27 000 *g*) in a JA20 rotor for 15 min at 4°C. The supernatant was loaded on a 0.8×11.6 cm TSK-Gel Toyopearl Butyl-650S column (Supelco, Bellefonte, PA) equilibrated with buffer B. After washing with buffer B, the column was eluted with a 10-ml linear gradient from buffer B to buffer A, followed by a 10-ml linear gradient from buffer A to water. 1-ml fractions were collected into tubes containing 25 μl 1 M Tris–HCl, pH 8.0. The MLCK-containing fractions were identified by SDS gel electrophoresis and pooled. Glycerol was added to a concentration of 20–50% and aliquots were stored at –70°C. The yield was typically ~12 mg.

Purification of N464K myosin

The mutant *Dictyostelium* myosin II N464K (Ruppel and Spudich, 1996), which is unable to hydrolyze ATP, was purified using a protocol adapted from Ruppel *et al.* (1994). *Dictyostelium* cells expressing N464K myosin were grown until confluent on 25×25 cm square plastic Petri plates containing 100 ml HL5:FM (10:3) and penicillin, streptomycin and 5 μg/ml G418. An additional 150 ml of media was then added to each plate, and the plates were shaken at 50 r.p.m. on a platform shaker for 2–3 days. All subsequent steps were performed on ice, and all centrifugation was done at 4°C. The cells were harvested at 1700 r.p.m. (820 *g*) in a Beckman JS-4.2 rotor for 20 min, and washed once in ice-cold 10 mM Tris–HCl, pH 7.5. 15 g of cells were used as the starting material. The cells were resuspended in 45 ml lysis buffer containing 25 mM HEPES–KOH, pH 7.4, 50 mM NaCl, 2 mM EDTA, 1 mM DTT and protease inhibitors (*p*-tosyl-L-arginine methyl ester, tosylphenylalanyl chloromethyl ketone, pepstatin, leupeptin, *o*-phenanthroline, PMSF, N^{α}-*p*-tosyl-L-lysine chloromethylketone and benzamidine). The cell suspension was dripped into liquid nitrogen, and stored at –70°C.

The frozen cells were lysed by adding 90 ml ice-cold lysis buffer and allowing them to thaw. The lysate was spun at 20 000 r.p.m. (48 000 *g*) in a JA20 rotor for 30 min. The pellet was resuspended in 45 ml 10 mM HEPES–KOH, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1 mM DTT, and then diluted with 90 ml of the same buffer without NaCl. The mixture was spun at 20 000 r.p.m. (48 000 *g*) in a JA20 rotor for 30 min, and the pellet resuspended in 60 ml 10 mM HEPES–KOH, pH 7.4, 300 mM NaCl, 3 mM $MgCl_2$, 2 mM ATP, 1 mM DTT and spun at 55 000 r.p.m. (300 000 *g*) for 1 h in a Beckman 60Ti rotor. 0.1 mM PMSF and 5 μg/ml boiled RNase A (bovine pancreatic type XII-A, Sigma Chemical Co, St Louis, MO) were added to the supernatant, which was then dialyzed against 10 mM piperazine-*N*,*N*′-bis[2-ethanesulfonic acid]–HCl (PIPES–HCl), pH 6.5, 10 mM $MgCl_2$, 50 mM NaCl and 1 mM DTT. The precipitate was collected by centrifugation at 20 000 r.p.m. (48 000 *g*) in a JA20 rotor for 30 min. The pellet was homogenized in 6.75 ml 10 mM HEPES–KOH, pH 7.4, 200 mM NaCl, 3 mM $MgCl_2$, 2 mM ATP, 1 mM DTT, and immediately spun at 55 000 r.p.m. (160 000 *g*) for 10 min in a Beckman TL100.3 rotor. The supernatant was diluted with 20.3 ml 10 mM PIPES–HCl, pH 6.5, 10 mM $MgCl_2$, 1 mM DTT, and spun at 55 000 r.p.m. (300 000 *g*) for 30 min in a 60Ti rotor.

The myosin recovered in the pellet was further purified by gel filtration on a 1.5×40 cm Bio-Gel A15m, 200–400 mesh column (Bio-Rad Laboratories, Richmond, CA), using a method modified from Griffith *et al.* (1987). This column removed contaminating actin, an unidentified 17 kDa protein, and contaminating protein phosphatase and heavy chain kinase activities. The column was equilibrated with 10 mM HEPES–KOH, pH 7.5, 200 mM NaCl, 1 mM DTT, and then preloaded with 11.6 ml 10 mM HEPES–KOH, pH 7.5, 200 mM NaCl, 1 mM DTT, 20 mM sodium pyrophosphate, pH 7.0, 0.6 M KI. The pellet was homogenized with 1 ml 10 mM HEPES–KOH, pH 7.5, 200 mM NaCl, 3 mM $MgCl_2$, 2 mM ATP, 1 mM DTT, and then diluted with 1 ml 10 mM HEPES–KOH, pH 7.5, 200 mM NaCl, 1 mM DTT, 40 mM sodium pyrophosphate, pH 7.0, 1.2 M KI, and immediately loaded on the column. Fractions containing pure myosin were located by electrophoresis on a 15% SDS–polyacrylamide gel. Filaments were formed from the pooled fractions by diluting with 3 volumes 10 mM PIPES–HCl, pH 6.5, 10 mM $MgCl_2$, 1 mM DTT. After incubating on ice for 1 h, the filaments were collected by centrifugation at 42 000 r.p.m. (210 000 *g*) in a Beckman 45Ti rotor for 2.5 h. The myosin pellet was redissolved in 1 ml 10 mM HEPES–KOH, pH 7.5, 200 mM NaCl, 1 mM DTT and aliquots were stored at –70°C. This preparation yielded myosin which was at least 80% unphosphorylated on the RLC.

Protein quantitation

Protein quantitation by the Bradford method (Bradford, 1976) was done as described in Smith (1987). The extinction coefficients for MLCK-A were determined as described in Scopes (1974) to be $E^{1\,mg/ml}_{278}=1.27$, $E^{1\,mg/ml}_{205}=31.8$. Based on this, measurements by the Bradford method using BSA as a standard were adjusted by a factor of 1.18. *Dictyostelium* myosin stocks were quantified by Bradford using rabbit skeletal myosin as a standard.

MLCK-A autophosphorylation site determination

0.37 mg MLCK-A was autophosphorylated in a 1 ml volume with 1 mM [γ-^{32}P]ATP (91 μCi/ml), 5 mM $MgCl_2$, 1 mM DTT, 25 mM Tris–HCl, pH 7.5 at 22°C for 1 h. The reaction was stopped by the addition of 50 μl 100% TCA, incubated on ice for 1 h, and centrifuged at top speed in a microcentrifuge at 4°C for 15 min. The pellet was dissolved in 0.5 ml 1.6 M urea, 80 mM Tris–HCl, pH 8.0. 1 μg endoproteinase AspN (sequencing grade, Boehringer Mannheim, Indianapolis, IN) dissolved in 0.1 ml 2 M urea, 100 mM Tris–HCl, pH 8.0 was added, and the digest was incubated overnight at 37°C. The sample was run on an Applied Biosystems (Foster City, CA) model 140A HPLC using a 2.1×150 mm C_{18} column (Vydac, Hesperia, CA). The peak containing the autophosphorylated peptide was identified by Cherenkov counting of the HPLC fractions. One major radioactive peak with an elution time of 26.5 min was identified. One-third of this fraction was sequenced on an Applied Biosystems model 477A automated sequencer.

For the autophosphorylation assays in Figure 1, 3 μg purified MLCK-A was autophosphorylated in a 10 μl reaction under the conditions described above, except that only labeled [γ-^{32}P]ATP (6000 Ci/mmol) was included. The reaction was stopped by the addition of an equal volume of 2× Laemmli sample buffer, and the sample was immediately heated to 95°C for 5 min.

Expression of mutant MLCK-As in Dictyostelium

The 1 kb *Sac*I–*Kpn*I fragment from pBCA15PT-SL′ (T.Q.P.Uyeda and J.A.Spudich, unpublished results) was cloned into *Sac*I–*Kpn*I-cut pBluescriptKS$^+$ (Stratagene, La Jolla, CA). This DNA fragment contains

the actin15 promoter and first eight amino acids of the actin15 coding sequence (to the *Hin*dIII site), a spacer region containing a *Bgl*II site, and the actin15 terminator. An *Nco*I site was placed at the start ATG of the actin15 coding sequence using the mutagenic oligonucleotide 5′-aaattaaataaaaaataa**cc**a**tgg**atggtg-3′, where the *Nco*I site is in bold, and mismatches with the template are underlined. This modified actin15 promoter–terminator cassette was inserted as a 1 kb *Sac*I–*Bam*HI fragment into the *Sac*I–*Bam*HI sites of pTIKL (T.Q.P.Uyeda and J.A. Spudich, unpublished results), a *Dictyostelium/E.coli* shuttle vector. This plasmid was digested with *Nco*I and *Bgl*II, and 0.9 kb *Nco*I–*Bam*HI DNA fragments containing mutant cDNA sequences of MLCK-A were inserted between the actin15 promoter and terminator. The T289A mutation was made as described above for the expression of T289A MLCK-A in *E.coli*. The T166A mutation was made using the antisense mutagenic oligonucleotide 5′-taccacatgcagcttgcattacaag-3′. The double mutant T166A, T289A was made by including both oligonucleotides in the mutagenesis reaction. The expression plasmids were introduced into MLCK-A$^-$ *Dictyostelium* (strain HS183; Smith *et al.*, 1996) by electroporation (Hadwiger and Firtel, 1992), and the cells were selected in HL5 media containing 5 μg/ml G418.

Phosphoamino acid analysis

MLCK-A immunoprecipitate from ^{32}P-labeled JH10 cells was run on an SDS–polyacrylamide gel and electrophoretically transferred to Immobilon membrane (Millipore, Bedford, MA). The MLCK-A band was placed in 200 μl constant boiling HCl (Pierce, Rockford, IL) in a screw-cap microfuge tube flushed with argon, and hydrolyzed for 2 h at 110°C. The phosphoamino acids were separated by thin-layer electrophoresis on Polygram Cel 300 plates (Macherey-Nagel, Duren, Germany) as described in Boyle *et al.* (1991), except that electrophoresis in the first dimension (pH 1.9) was carried out for 40 min.

MLCK-A assays

MLCK-A activity was measured at 22°C in 25 mM Tris–HCl, pH 7.5, 5 mM HEPES–KOH, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 1 mg/ml BSA, 1.5 mM DTT, 1 mM [γ-^{32}P]ATP (0.1 μCi/μl) and MLCK-A [3.3 μg/ml (100 nM) for wild-type and 0.33 μg/ml (10 nM) for T166E]. Reaction aliquots (taken over 30 min) were stopped by the addition of an equal volume of SDS sample buffer, and heated to 95°C for 5 min. The samples were electrophoresed on a 15% SDS–polyacrylamide gel, and incorporation of ^{32}P into the RLC was quantified using a Molecular Dynamics (Sunnyvale, CA) PhosphorImager.

Other methods

Procedures for metabolic labeling *Dictyostelium* cells and for immunoprecipitation of MLCK-A are described elsewhere (Smith *et al.*, 1996). For conA treatment of metabolically labeled cells, the labeled cells were washed once in 20 mM MES–KOH, pH 6.8, 20 mM NaCl, 1 mM EDTA, then resuspended in 180 μl of the same buffer and split into two tubes. ConA (Sigma Chemical Co., St Louis, MO) was added to a final concentration of 30 μg/ml to one tube. After 1 min, 10 μl 10% SDS was added, and the MLCK-A was immunoprecipitated.

Acknowledgements

We thank Al Smith and Jim Kenny (Beckman Center PAN Facility, Stanford) for phosphopeptide purification and sequencing. We also thank Jonathan Goldberg for helpful discussions, and James Sabry for critically reading this manuscript. L.A.S. was supported by a Muscular Dystrophy Association postdoctoral fellowship, and J.L.S. was supported by postdoctoral fellowships from the Muscular Dystrophy Association and the American Heart Association. This work was supported by NIH grant GM46551 to J.A.S.

References

Ahn,N.G., Segar,R., Bratlein,R.L., Diltz,C.D., Tonks,W.K. and Krebs, E.G. (1991) Multiple components in an epidermal growth factor-stimulated protein kinase cascade. *In vitro* activation of a myelin basic protein/microtubule-associated protein kinase 2 kinase. *J. Biol. Chem.*, **266**, 4220–4227.

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

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

Bossemeyer,D., Engh,R.A., Kinzel,V., Ponstingl,H. and Huber,R. (1993) Phosphotransferase and substrate binding mechanism of the cAMP-dependent protein kinase catalytic subunit from porcine heart as deduced from the 2.0 Å structure of the complex with Mn^{2+} adenylyl imidodiphosphate and inhibitor peptide PKI(5-24). *EMBO J.*, **12**, 849–859.

Boyle,W.J., van der Geer,P. and Hunter,T. (1991) Phosphopeptide mapping and phosphoamino acid analysis by two-dimensional separation on thin-layer cellulose plates. In Hunter,T. and Sefton,B.M. (eds), *Protein Phosphorylation. Part B. Analysis of Protein Phosphorylation, Protein Kinase Inhibitors, and Protein Phosphatases*. Academic Press, San Diego, pp. 110–149.

Bradford,M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.*, **72**, 248–254.

Chen,P., Ostrow,B.D., Tafuri,S.R. and Chisholm,R.L. (1994) Targeted disruption of the *Dictyostelium* RMLC gene produces cells defective in cytokinesis and development. *J. Cell Biol.*, **127**, 1933–1944.

Chen,T.L., Kowalczyk,P.A., Ho,G. and Chisholm,R.L. (1995) Targeted disruption of the *Dictyostelium* myosin essential light chain gene produces cells defective in cytokinesis and morphogenesis. *J. Cell Sci.*, **108**, 3207–3218.

Cohen,S.M., Knecht,D., Lodish,H.F. and Loomis,W.F. (1986) DNA sequences required for expression of a *Dictyostelium* actin gene. *EMBO J.*, **5**, 3361–3366.

Cox,S., Radzio-Andzelm,E. and Taylor,S.S. (1994) Domain movements in protein kinases. *Curr. Opin. Struct. Biol.*, **4**, 893–901.

De Bondt,H., Rosenblatt,J., Jancarik,J., Jones,H.D., Morgan,D.O. and Kim,S.-H. (1993) Crystal structure of cyclin-dependent kinase 2. *Nature*, **363**, 595–602.

De Lozanne,A. and Spudich,J.A. (1987) Disruption of the *Dictyostelium* myosin heavy chain gene by homologous recombination. *Science*, **236**, 1086–1091.

Ducommun,B., Brambilla,P., Felix,M.-A., Franza,B.R.J., Karsenti,E. and Draetta,G. (1991) cdc2 phosphorylation is required for its interaction with cyclin. *EMBO J.*, **10**, 3311–3319.

Egelhoff,T.T., Lee,R.J. and Spudich,J.A. (1993) Dictyostelium myosin heavy chain phosphorylation sites regulate myosin filament assembly and localization *in vivo*. *Cell*, **75**, 363–371.

Goldberg,J., Nairn,A.C. and Kuriyan,J. (1996) Structural basis for the autoinhibition of calcium/calmodulin-dependent protein kinase I. *Cell*, **84**, 875–887.

Goldsmith,E.J. and Cobb,M.H. (1994) Protein kinases. *Curr. Opin. Struct. Biol.*, **4**, 833–840.

Gould,K.L., Moreno,S., Owen,D.J., Sazer,S. and Nurse,P. (1991) Phosphorylation at Thr167 is required for *Schizosaccharomyces pombe* p34^{cdc2} function. *EMBO J.*, **10**, 3297–3309.

Griffith,L.M., Downs,S.M. and Spudich,J.A. (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.

Hadwiger,J.A. and Firtel,R.A. (1992) Analysis of Gα4, a G-protein subunit required for multicellular development in *Dictyostelium*. *Genes Dev.*, **6**, 38–49.

Hammer,J.A.,III (1994) Regulation of *Dictyostelium* myosin II by phosphorylation: what is essential and what is important? *J. Cell Biol.*, **127**, 1779–1782.

Hanks,S.K. and Hunter,T. (1995) The eukaryotic protein kinase superfamily. In Hardie,G. and Hanks,S. (eds), *The Protein Kinase Facts Book. Protein-Serine Kinases*. Academic Press, San Diego, CA, pp. 7–47.

Hu,S.-H., Parker,M.W., Lei,J.Y., Wilce,M.C.J., Benian,G.M. and Kemp,B.E. (1994) Insights into autoregulation from the crystal structure of twitchin kinase. *Nature*, **369**, 581–584.

Huang,W. and Erickson,R.L. (1994) Constitutive activation of MEK1 by mutation of serine phosphorylation sites. *Proc. Natl Acad. Sci. USA*, **91**, 8960–8963.

Hubbard,S.R., Wei,L., Ellis,L. and Hendrickson,W.A. (1994) Crystal structure of the tyrosine kinase domain of the human insulin receptor. *Nature*, **372**, 746–753.

Hughes,K., Nikolakaki,E., Plyte,S.E., Totty,N.F. and Woodgett,J.R. (1993) Modulation of the glycogen synthase kinase-3 family by tyrosine phosphorylation. *EMBO J.*, **12**, 803–808.

Johnson,L.N., Noble,M.E.M. and Owen,D.J. (1996) Active and inactive protein kinases: structural basis for regulation. *Cell*, **85**, 149–158.

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

Knighton,D.R., Zheng,J., Ten Eyck,L.F., Ashford,V.A., Xuong,N.-H., Taylor,S.S. and Sowadski,J.M. (1991a) Crystal structure of the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. *Science*, **253**, 404–414.

Knighton,D.R., Zheng,J., Ten Eyck,L.F., Xuong,N.-H., Taylor,S.S. and Sowadski,J.M. (1991b) Structure of a peptide inhibitor bound to the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. *Science*, **253**, 414–420.

Knighton,D.R., Bell,S.M., Zheng,J., Ten Eyck,L.F., Xuong,N.-H., Taylor,S.S. and Sowadski,J.M. (1993) 2.0Å refined crystal structure of the catalytic subunit of cAMP-dependent protein kinase complexed with a peptide inhibitor and detergent. *Acta Crystallogr.*, **D49**, 357–361.

Kunkel,T.A. (1987) Oligonucleotide-directed mutagenesis without phenotypic selection. In Ausubel,F.M., Brent,R., Kingston,R.E., Moore,D.D., Seidman,J.G., Smith,J.A. and Struhl,K. (eds), *Current Protocols in Molecular Biology*. John Wiley & Sons, New York, pp. 8.1.1–8.1.6.

Lee,J.C. and Edelman,A.M. (1995) Activation of Ca^{2+}-calmodulin-dependent protein kinase Ia is due to direct phosphorylation by its activator. *Biochem. Biophys. Res. Commun.*, **210**, 631–637.

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

Mochizuki,H., Sugita,R., Ito,T. and Hidaka,H. (1993) Phosphorylation of Ca^{2+}/calmodulin-dependent protein kinase V and regulation of its activity. *Biochem. Biophys. Res. Commun.*, **197**, 1595–1600.

Morgan,D.O. and De Bondt,H.L. (1994) Protein kinase regulation: insights from crystal structure analysis. *Curr. Opin. Cell Biol.*, **6**, 239–246.

Noegel,A.A. and Luna,J.E. (1995) The *Dictyostelium* cytoskeleton. *Experientia*, **51**, 1135–1143.

Orr,J.W. and Newton,A.C. (1994) Requirement for negative charge on 'activation loop' of protein kinase C. *J. Biol. Chem.*, **269**, 27715–27718.

Ostrow,B.D., Chen,P. and Chisholm,R.L. (1994) Expression of a myosin regulatory light chain phosphorylation site mutant complements the cytokinesis and developmental defects of *Dictyostelium* RMLC null cells. *J. Biol. Chem.*, **127**, 1945–1955.

Owen,D.J., Noble,M.E., Garman,E.F., Papageoriou,A.C. and Johnson,L.N. (1995) Two structures of the catalytic domain of phosphorylase kinase: an active protein kinase complexed with a substrate analogue and product. *Structure*, **3**, 467–482.

Pasternak,C., Spudich,J.A. and Elson,E.L. (1989) Capping of surface receptors and concomitant cortical tension are generated by conventional myosin. *Nature*, **341**, 549–551.

Payne,D.M., Rossomando,A.J., Martino,P., Erickson,A.K., Her,J.-H., Shabanowitz,J., Hunt,D.F., Weber,M.J. and Sturgill,T.W. (1991) Identification of the regulatory sites in pp42/mitogen-activated protein kinase (MAP kinase). *EMBO J.*, **10**, 885–892.

Picciotto,M.R., Czernik,A.J. and Nairn,A.C. (1993) Calcium/calmodulin-dependent protein kinase I. cDNA cloning and identification of autophosphorylation site. *J. Biol. Chem.*, **268**, 26512–26521.

Pollenz,R.S., Chen,T.L., Trivinos-Lagos,L. and Chisholm,R.L. (1992) The *Dictyostelium* essential light chain is required for myosin function. *Cell*, **69**, 951–962.

Robbins,D.J., Zhen,E., Owaki,H., Vanderbilt,C.A., Ebert,D., Geppert, T.D. and Cobb,M.H. (1993) Regulation and properties of extracellular signal regulated protein kinases 1 and 2 *in vitro*. *J. Biol. Chem.*, **268**, 5097–5106.

Ruppel,K.M. and Spudich,J.A. (1996) Structure-function studies of the myosin motor domain: importance of the 50 kDa cleft. *Mol. Biol. Cell*, **7**, 1123–1136.

Ruppel,K.M., Uyeda,T.Q.P. and Spudich,J.A. (1994) Role of highly conserved lysine 130 of myosin motor domain. *J. Biol. Chem.*, **269**, 18773–18780.

Scopes,R.K. (1974) Measurement of protein by spectrophotometry at 205 nm. *Anal. Biochem.*, **59**, 277–282.

Shoji,S., Ericsson,L.H., Walsh,D.A., Fischer,E.H. and Titani,K. (1983) Sequence of two phosphorylated sites in the catalytic subunit of bovine cardiac muscle adenosine 3′:5′-monophosphate-dependent protein kinase. *Biochemistry*, **22**, 3702–3709.

Smith,J.A. (1987) Quantitation of proteins. In Ausubel,F.M., Brent,R., Kingston,R.E., Moore,D.D., Seidman,J.G., Smith,J.A. and Struhl,K. (eds), *Current Protocols in Molecular Biology*. John Wiley & Sons, pp. 10.1.1–10.1.2.

Smith,J.L., Silveira,L.A. and Spudich,J.A. Myosin light chain kinase gene disruption in *Dictyostelium*: a role for MLCK-A in cytokinesis and evidence for multiple MLCKs. *Proc. Natl Acad. Sci. USA*, in press.

Solomon,M.J., Lee,T. and Kirschner,M.W. (1992) Role of phosphorylation in $p34^{cdc2}$ activation: identification of an activating kinase. *Mol. Biol. Cell*, **3**, 13–27.

Steinberg,R.A., Cauthron,R.D., Symcox,M.M. and Shuntoh,H. (1993) Autoactivation of catalytic (Cα) subunit of cyclic AMP-dependent protein kinase by phosphorylation of threonine 197. *Mol. Cell. Biol.*, **13**, 2332–2341.

Stull,J.T., Nunnally,M.H. and Michnoff,C.H. (1986) Calmodulin-dependent protein kinases. In Boyer,P.D. and Krebs,E.G. (eds), *Control by Phosphorylation, Part A*. Academic Press, Orlando, pp. 113–166.

Sweeney,H.L., Yang,Z., Zhi,G., Stull,J.T. and Trybus,K.M. (1994). Charge replacement near the phosphorylatable serine of the myosin regulatory light chain mimics aspects of phosphorylation. *Proc. Natl Acad. Sci. USA*, **91**, 1490–1494.

Tafuri,S.R., Rushforth,A.M., Kuczmarski,E.R. and Chisholm,R.L. (1989) *Dictyostelium discoideum* myosin: Isolation and characterization of cDNAs encoding the regulatory light chain. *Mol. Cell. Biol.*, **9**, 3073–3080.

Tan,J.L. and Spudich,J.A. (1990) *Dictyostelium* myosin light chain kinase: purification and characterization. *J. Biol. Chem.*, **265**, 13818–13824.

Tan,J.L. and Spudich,J.A. (1991) Characterization and bacterial expression of the *Dictyostelium* myosin light chain kinase cDNA: identification of an autoinhibitory domain. *J. Biol. Chem.*, **266**, 16044–16049.

Tan,J.L., Ravid,S. and Spudich,J.A. (1992) Control of nonmuscle myosins by phosphorylation. *Annu. Rev. Biochem.*, **61**, 721–759.

Thorsness,P.E. and Koshland,D.E.,Jr (1987) Inactivation of isocitrate dehydrogenase by phosphorylation is mediated by the negative charge of the phosphate. *J. Biol. Chem.*, **262**, 10422–10425.

Wessels,D., Soll,D.R., Knecht,D., Loomis,W.F., De Lozanne,A. and Spudich,J. (1988) Cell motility and chemotaxis in *Dictyostelium* amebae lacking myosin heavy chain. *Dev. Biol.*, **128**, 164–177.

White,M.F., Shoelson,S.E., Keutmann,H. and Kahn,C.R. (1988) A cascade of tyrosine autophosphorylation in the beta-subunit activates the phosphotransferase of the insulin receptor. *J. Biol. Chem.*, **263**, 2969–2980.

Xu,R.-M., Carmel,G., Sweet,R.M., Kuret,J. and Cheng,X. (1995) Crystal structure of casein kinase-1, a phosphate directed protein kinase. *EMBO J.*, **14**, 1015–1023.

Yan,M. and Templeton,D.J. (1994) Identification of 2 serine residues of MEK-1 that are differentially phosphorylated during activation by raf and MEK kinase. *J. Biol. Chem.*, **269**, 19067–19073.

Yonemoto,W., Garrod,S.M., Bell,S.M. and Taylor,S.S. (1993) Identification of phosphorylation sites in the recombinant catalytic subunit of cAMP-dependent protein kinases. *J. Biol. Chem.*, **268**, 18626–18632.

Zhang,R., Strand,A., Robbins,D., Cobb,M.H. and Goldsmith,E.J. (1994) Atomic structure of the MAP kinase ERK2 at 2.3 Å resolution. *Nature*, **367**, 704–710.

Zheng,J., Knighton,D.R., Xuong,N.H., Taylor,S.S., Sowadski,J.M. and Ten Eyck,L.F. (1993) Crystal structures of the myristylated catalytic subunit of cAMP-dependent protein kinase reveal open and closed conformations. *Protein Sci.*, **2**, 1559–1573.

Zhi,G., Herring,B.P. and Stull,J.T. (1994) Structural requirements for phosphorylation of myosin regulatory light chain from smooth muscle. *J. Biol. Chem.*, **269**, 24723–24727.

Received on June 24, 1996; revised on July 31, 1996