In this study, we examined the activation mechanism of Dictyostelium myosin light chain kinase A (MLCK-A) using constitutively active Ca\(^{2+}\)/calmodulin-dependent protein kinase kinase as a surrogate MLCK-A kinase. MLCK-A was phosphorylated at Thr\(^{166}\) by constitutively active Ca\(^{2+}\)/calmodulin-dependent protein kinase kinase, resulting in an ~140-fold increase in catalytic activity, using intact Dictyostelium myosin II. Recombinant Dictyostelium myosin II regulatory light chain and Kemptamide were also readily phosphorylated by activated MLCK-A. Mass spectrometry analysis revealed that MLCK-A expressed by Escherichia coli was auto-phosphorylated at Thr\(^{289}\) and that, subsequent to Thr\(^{166}\) phosphorylation, MLCK-A also underwent a slow rate of autophosphorylation at multiple Ser residues. Using site-directed mutagenesis, we show that autophosphorylation at Thr\(^{289}\) is required for efficient phosphorylation and activation by an upstream kinase. By performing enzyme kinetics analysis on a series of MLCK-A truncation mutants, we found that residues 283-288 function as an autoinhibitory domain and that autoinhibition is fully relieved by Thr\(^{166}\) phosphorylation. Simple removal of this region resulted in a significant increase in the \(k_{\text{cat}}\) of MLCK-A; however, it did not generate maximum enzymatic activity. Together with the results of our kinetic analysis of the enzymes, these findings demonstrate that Thr\(^{166}\) phosphorylation of MLCK-A by an upstream kinase subsequent to autophosphorylation at Thr\(^{289}\) results in generation of maximum MLCK-A activity through both release of an autoinhibitory domain from its catalytic core and a further increase (15–19-fold) in the \(k_{\text{cat}}\) of the enzyme.

Conventional myosin light chain kinase (MLCK),\(^1\) which belongs to the family of Ca\(^{2+}\)/calmodulin (CaM)-dependent protein kinases, induces a high degree of myosin motor activity by specifically phosphorylating the myosin II regulatory light chain (MRLC) (1–4). Myosin II phosphorylation has been shown to be involved in smooth muscle contraction and cell motility in non-muscle cells (5–7). In Dictyostelium discoideum myosin II, phosphorylation of MRLC at Ser\(^{13}\) induces its actin-dependent ATPase activity and its motor activity (8–11). A recent study indicated that filamentous assembly is required for efficient regulation of Dictyostelium myosin II by MRLC phosphorylation (12). In contrast to vertebrate MLCK, an unconventional MLCK (MLCK-A) has been identified and characterized in Dictyostelium and shown not to require Ca\(^{2+}\)/CaM for its activity (13, 14). The results of a genetic study using cells lacking MLCK-A indicate that MLCK-A plays a role in efficient cytokinesis (15). It has been shown that MLCK-A undergoes autophosphorylation at Thr\(^{289}\), resulting in increased activity of the enzyme (13); but autophosphorylation is not required for MLCK-A activity in vivo (16). Thus, the role of autophosphorylation at Thr\(^{289}\) remains unclear. With regard to the activation mechanism of this enzyme, MLCK-A has been shown to be activated and phosphorylated by concanavalin A treatment of cells (15). This activation is likely due to phosphorylation at Thr\(^{166}\) in the activation loop because the activity of the T166E mutant is 12-fold higher than that of wild-type MLCK-A (17). Interestingly, cGMP activates endogenous MLCK-A in cell lysates, suggesting that MLCK-A kinase can be either directly or indirectly up-regulated by cGMP signals and thereby induce phosphorylation and activation of MLCK-A (16). Such activation would be analogous to the activation mechanism of protein kinase cascades such as the mitogen-activated protein kinase cascade (18) and the CaM kinase cascade. For example, the multifunctional Ca\(^{2+}\)/CaM-dependent protein kinases (CaM-Ks), CaM-KI and CaM-KIV, are activated through phosphorylation at the Thr residue in the activation loop by an upstream kinase, Ca\(^{2+}\)/CaM-dependent protein kinase kinase (CaM-KK) (19, 20).

Because neither the MLCK-A kinase nor MLCK-A phosphorylated at Thr\(^{166}\) has been investigated, the regulatory mechanism of MLCK-A remains unclear. Here, we found that mammalian CaM-KK, an activating kinase for CaM-KI and CaM-KIV, was capable of phosphorylating Thr\(^{166}\) of MLCK-A, resulting in a large increase in the catalytic efficiency of MLCK-A. This allowed us to examine several aspects of MLCK-A activation, including the role of autophosphorylation at Thr\(^{289}\), the specific location of the autoinhibitory region, and the relationship between Thr\(^{166}\) phosphorylation and the autoinhibitory mechanism of MLCK-A.
Comparison of the amino acid sequences of Dictyostelium MLCK-A and C. elegans and human CaM-KI. Shown is a comparison of the amino acid sequences of Dictyostelium (Dict.) MLCK-A (GenBank™/EMBL accession number M64176) (14), C. elegans (C.ele.) CaM-KI (accession number AB021864) (28), and human CaM-KI (accession number L41816) (27). Identical residues are indicated by black boxes, and gaps in the alignment are indicated by dashes. The asterisk indicates Thr in the activation loop of MLCK. The putative regulatory domain, including an autoinhibitory domain (AID; solid underlines) and a CaM-binding domain (CBD; dashed underlines), in CaM-KI is indicated.

**EXPERIMENTAL PROCEDURES**

**Materials**—CaM-KKs cDNA (GenBank™/EMBL accession number L42810) (21) was obtained from a rat brain cDNA library. Constitutively active CaM-KKa (CaM-KKc; GST-CaM-KK (84–434)) was constructed and expressed in Escherichia coli JM109 and purified by glutathione-Sepharose column chromatography (22). Dictyostelium myosin II was prepared according to Ruppel et al. (11). Recombinant His-tagged Dictyostelium MRLC was prepared as described previously (9). All other chemicals were from standard commercial sources.

**Construction and Expression of Dictyostelium MLCK-A—**GST-fused wild-type MLCK-A and mutants were constructed by amplification of cDNA fragments using pET-MLCK-A (17) as a template, a sense primer wild-type MLCK-A and mutants were constructed by amplification of previously (9). All other chemicals were from standard commercial sources.

**Activation of Dictyostelium MLCK-A by CaM-KKc**—Activation of Dictyostelium MLCK-A was performed by adding CaM-KKc to the MLCK-A reaction mixture and measuring MRLC phosphorylation by Cerenkov counting of the excised gels. Phosphate incorporation into MRLC by Cerenkov counting of the excised gels. Phosphate incorporation into Kemptamide was determined by liquid scintillation counting of the filters. To determine the kinetic parameters of MLCK-A, we used 467 μM Kemptamide and 50 μM [γ-32P]ATP (500–1000 cpm/pmol) in the presence of 1 ng/ml activated wild-type MLCK-A and MLCK-A-(1–277), and 1.5 μg/ml activated wild-type MLCK-A and MLCK-A-(1–277) for Kemptamide phosphorylation or 20 μg/ml wild-type MLCK-A, 20 μg/ml MLCK-A-(1–277), and 0.15 μg/ml activated wild-type MLCK-A and MLCK-A-(1–277) for MRLC phosphorylation.

**Phosphorylation of Dictyostelium MLCK-A by CaM-KKc—**Purified recombinant MLCK-A (0.16 mg/ml) were assayed at 30 °C for the indicated times in a solution containing 50 mM HEPES (pH 7.5), 10 mM MgAc2, 1 mM DTT, 1 mM EGTA, and 200 μM ATP in either the presence or absence of 6.6 μg/ml CaM-KKc. The reaction was initiated by the addition of ATP and terminated by 21-fold dilution with 50 mM HEPES (pH 7.5), 2 mg/ml bovine serum albumin, 10% ethylene glycol, and 1 mM EDTA. Five μl of the diluted sample (37.5 ng of MLCK-A) was then subjected to the protein kinase assay.

**MLCK-A Activity Assay—**MLCK-A activity was measured at 30 °C for 5–10 min (or for 2 min for His-tagged MLCK-A) in a solution (25 μl) containing 50 mM HEPES (pH 7.5), 10 mM MgAc2, 1 mM DTT, 40 μM smooth muscle MRLC peptide (Kemptamide, Lys-Lys-Arg-Pro-Gln-Arg-Ala-Thr-Ser-Asn-Val-Phe-Ser-NH2) (24) or various concentrations of recombinant Dictyostelium MRLC, and 400 μM [γ-32P]ATP (~1000 cpm/pmol) in the presence of 1 ng/ml activated wild-type MLCK-A. The reaction was initiated by the addition of [γ-32P]ATP and terminated either by spotting aliquots (15 μl) onto phosphocellulose paper (Whatman P-81), followed by several washes with 75 mM phosphoric acid (25) for peptide phosphorylation, or by the addition of SDS-PAGE sample buffer, and the samples were then subjected to SDS–15% PAGE, followed by quantifying 32P incorporation into MRLC by Cerenkov counting of the excised gels. Phosphate incorporation into Kemptamide was determined by liquid scintillation counting of the filters. To determine the kinetic parameters of MLCK-A, we used 467 μM Kemptamide and 50–400 μM [γ-32P]ATP for titration of ATP. For titration of Kemptamide or MRLC, 400 μM [γ-32P]ATP and 29–467 μM Kemptamide or 6–24 μM MRLC were used. The enzyme concentrations used for measurement of the kinetic parameters were 64 μg/ml wild-type MLCK-A, 20 μg/ml MLCK-A-(1–277), and 1.5 μg/ml activated wild-type MLCK-A and MLCK-A-(1–277) for Kemptamide phosphorylation or 20 μg/ml wild-type MLCK-A, 2.2 μg/ml MLCK-A-(1–277), and 0.15 μg/ml activated wild-type MLCK-A and MLCK-A-(1–277) for MRLC phosphorylation.

**Phosphorylation of Dictyostelium MLCK-A by CaM-KKc—**Purified recombinant MLCK-A (0.16 mg/ml) were assayed at 30 °C for the indicated times in a solution containing 50 mM HEPES (pH 7.5), 10 mM MgAc2, 1 mM DTT, 1 mM EGTA, and 200 μM [γ-32P]ATP (500–1000 cpm/pmol) in the presence or absence of 6.6 μg/ml CaM-KKc. The reaction was initiated by the addition of [γ-32P]ATP and terminated either by spotting 15 μl of the sample onto P-81 paper, followed by measurement of 32P incorporation into MLCK-A as described above, or by the addition of the SDS-PAGE sample buffer. The samples were then subjected to SDS–10% PAGE, followed by autoradiography.

**Dictyostelium Myosin II Phosphorylation—**Purified Dictyostelium myosin II (0.2–1 mg/ml) was incubated with either activated or unactivated MLCK-A (1.5 μg/ml) at 30 °C for the indicated times in a solution containing 50 mM HEPES (pH 7.5), 10 mM MgAc2, 1 mM DTT, 1 mM EGTA, and 200 μM ATP in either the presence or absence of 6.6 μg/ml CaM-KKc. The reaction was initiated by the addition of ATP and terminated by 21-fold dilution with 50 mM HEPES (pH 7.5), 2 mg/ml bovine serum albumin, 10% ethylene glycol, and 1 mM EDTA. Five μl of the diluted sample (37.5 ng of MLCK-A) was then subjected to the protein kinase assay.

**MLCK-A Activity Assay—**MLCK-A activity was measured at 30 °C for 5–10 min (or for 2 min for His-tagged MLCK-A) in a solution (25 μl) containing 50 mM HEPES (pH 7.5), 10 mM MgAc2, 1 mM DTT, 40 μM smooth muscle MRLC peptide (Kemptamide, Lys-Lys-Arg-Pro-Gln-Arg-Ala-Thr-Ser-Asn-Val-Phe-Ser-NH2) (24) or various concentrations of recombinant Dictyostelium MRLC, and 400 μM [γ-32P]ATP (~1000 cpm/pmol) in the presence of 1 ng/ml activated wild-type MLCK-A. The reaction was initiated by the addition of [γ-32P]ATP and terminated either by spotting aliquots (15 μl) onto phosphocellulose paper (Whatman P-81), followed by several washes with 75 mM phosphoric acid (25) for peptide phosphorylation, or by the addition of SDS-PAGE sample buffer, and the samples were then subjected to SDS–15% PAGE, followed by quantifying 32P incorporation into MRLC by Cerenkov counting of the excised gels. Phosphate incorporation into Kemptamide was determined by liquid scintillation counting of the filters. To determine the kinetic parameters of MLCK-A, we used 467 μM Kemptamide and 50–400 μM [γ-32P]ATP for titration of ATP. For titration of Kemptamide or MRLC, 400 μM [γ-32P]ATP and 29–467 μM Kemptamide or 6–24 μM MRLC were used. The enzyme concentrations used for measurement of the kinetic parameters were 64 μg/ml wild-type MLCK-A, 20 μg/ml MLCK-A-(1–277), and 1.5 μg/ml activated wild-type MLCK-A and MLCK-A-(1–277) for Kemptamide phosphorylation or 20 μg/ml wild-type MLCK-A, 2.2 μg/ml MLCK-A-(1–277), and 0.15 μg/ml activated wild-type MLCK-A and MLCK-A-(1–277) for MRLC phosphorylation.

**Phosphorylation of Dictyostelium MLCK-A by CaM-KKc—**Purified recombinant MLCK-A (0.16 mg/ml) were assayed at 30 °C for the indicated times in a solution containing 50 mM HEPES (pH 7.5), 10 mM MgAc2, 1 mM DTT, 1 mM EGTA, and 200 μM [γ-32P]ATP (500–1000 cpm/pmol) in the presence or absence of 6.6 μg/ml CaM-KKc. The reaction was initiated by the addition of [γ-32P]ATP and terminated either by spotting 15 μl of the sample onto P-81 paper, followed by measurement of 32P incorporation into MLCK-A as described above, or by the addition of the SDS-PAGE sample buffer. The samples were then subjected to SDS–10% PAGE, followed by autoradiography.

**Dictyostelium Myosin II Phosphorylation—**Purified Dictyostelium myosin II (0.2–1 mg/ml) was incubated with either activated or unactivated MLCK-A (1.5 μg/ml) at 30 °C for the indicated times in a
solution containing 150 mM NaCl, 50 mM HEPES, 10 mM Mg(Ac)₂, 1 mM DTT, 1 mM EGTA, 0.4 mg/ml bovine serum albumin, and 1 mM \([\gamma-32P]ATP\). The reaction was terminated by the addition of SDS-PAGE sample buffer, and the samples were then subjected to SDS-15% PAGE, followed by either autoradiography or quantifying 32P incorporation into MRLC by Cerenkov counting of the excised gels.

**Identification of Phosphorylation Sites in Dictyostelium MLCK-A by Mass Spectrometry**—Either purified recombinant MLCK-A (10 μg) or MLCK-A (10 μg) phosphorylated by incubation with CaM-KKc for 120 min as described above was separated by SDS-10% PAGE, followed by in-gel digestion with 17 μg/ml chymotrypsin (Roche Applied Science) overnight at 37 °C. The digested peptides were eluted by 0.1% formic acid and subjected to liquid chromatography-tandem mass spectrometry (MS/MS) analysis. Liquid chromatography-MS/MS analysis was performed using a Micromass Q-ToF2 quadrupole/time-of-flight hybrid mass spectrometer interfaced with a Micromass CapLC²⁵⁰ capillary reverse-phase liquid chromatography system. A 90-min linear gradient from 5 to 45% acetonitrile in 0.1% formic acid was produced and split at a 1:20 ratio, and the gradient solution was injected into a PepMap C₁₅ Nano LC column (75 μm × 150 mm; LC Packings, Amsterdam, The Netherlands) at ~25 nL/min. The eluted peptides were sprayed directly into the mass spectrometer. MS/MS data were acquired using Micromass MassLynx software and converted to a single text file (containing the observed m/z of the precursor peptide and fragment ion m/z and intensity values) using Micromass ProteinLynx software. The file was analyzed with the Mascot MS/MS Ions search (Matrix Science)² to assign non-phosphorylated and phosphorylated peptides to Dictyostelium MLCK-A amino acid sequence (GenBank®/EBI accession number Z41001).

**TABLE I**

| Peptide | Observed phosphopeptide mass | MLCK-A residues | Inferred peptide sequence |
|---------|------------------------------|-----------------|---------------------------|
| A-1     | 1635.82                      | 283–295         | IVERQpKTQTKLN              |
| B-1     | 994.51                       | 247–254         | VVDpSKKRL                  |
| B-2     | 1108.80                      | 153–162         | GLpSKIGQYT                  |
| B-3     | 1635.88                      | 283–295         | IVERQpKTQTKLN              |
| B-4     | 1901.84                      | 163–179         | VMqTpACpTSYVAF               |
| B-5     | 2141.13                      | 35–52           | AIlKpVINKpSELGKDYEK              |
| B-6     | 2178.01                      | 265–282         | LSNNpSMNTIDTVKMKEY            |

A single phosphopeptide (A-1) was derived from purified Dictyostelium MLCK-A. Phosphopeptides B-1–6 were derived from Dictyostelium MLCK-A incubated with CaM-KKc for 120 min in the presence of 200 μM ATP as described in the legend to Fig. 2B.
FIG. 3. Identification of phosphorylation sites in Dictyostelium MLCK-A. Purified recombinant MLCK-A (A) and MLCK-A activated by CaM-KKc for 120 min as described in the legend to Fig. 2A (B) were digested with chymotrypsin and then subjected to liquid chromatography-MS/MS analysis as shown in Table I. The singly charged ion of a peptide (A-1, residues 283–295) derived from untreated MLCK-A (A) and that of a peptide (B-4, residues 163–179) derived from activated MLCK-A (B) were subjected to MS/MS analysis as described under "Experimental Procedures." The observed fragment ions are indicated above and below each peptide sequence. The phosphorylated Thr residues are indicated. Cys*, carboxymethylcysteine.
Regulatory Mechanism of Dictyostelium MLCK-A

Fig. 4. Phosphorylation and activation of Dictyostelium MLCK-A mutants. A, recombinant MLCK-A mutants (0.16 mg/ml) and the wild-type enzyme (WT) were incubated at 30 °C for 30 min in the solution described in the legend to Fig. 2A in the presence (+) or absence (−) of 6.6 μg/ml CaM-KKc. The reaction was terminated by the addition of SDS-PAGE sample buffer. The samples were then subjected to SDS-10% PAGE, stained with Coomassie Brilliant Blue (upper panel), and subjected to autoradiography (lower panel). Arrows indicate MLCK-A. B, recombinant MLCK-A mutants (0.16 mg/ml) and the wild-type enzyme were incubated at 30 °C for 30 min as described for A with 200 μM ATP in the presence (+) or absence (−) of 6.6 μg/ml CaM-KKc. The reaction was terminated, and MLCK-A (1.5 μg/ml) was then subjected to the protein kinase assay at 30 °C for 10 min in the presence of 400 μM [γ-32P]ATP using 40 μM Kemptide as a substrate as described under "Experimental Procedures." Results represent means ± S.E. of three experiments. C, either wild-type MLCK-A or the T289A mutant was incubated with (+) or without (−) protein phosphatase 2A (PP2A) at 30 °C for 60 min and then analyzed by SDS-7.5% PAGE. Results are representative of three independent experiments. D, shown is the activation of MLCK-A Thr289 mutants by CaM-KKc. Wild-type MLCK-A (•), as shown in Fig. 2B, the T289A mutant (○), or the T289E mutant (□) at 0.16 mg/ml was incubated with 200 μM ATP at 30 °C for the indicated times (0–90 min) in the presence of 6.6 μg/ml CaM-KKc as described in the legend to Fig. 2B. The reaction was terminated, and MLCK-A (1.5 μg/ml) was then subjected to the protein kinase assay as described for B. Results for the MLCK-A Thr289 mutants represent triplicate experiments.

M641761. We set search parameters as follows: data base, NCBI nr; taxonomy, Dictyostelium discoideum; enzyme, none; fixed modifications, carbamyldimethyl; variable modifications, phospho (Ser/Thr); peptide tolerance, ±0.2 Da; MS/MS tolerance, ±0.2 Da; and peptide charge, 1+, 2+, and 3+.

Protein Phosphatase 2A Treatment of Dictyostelium MLCK-A—Either wild-type MLCK-A (3.5 μg) or the T289A mutant (1.2 μg) was incubated with or without 0.025% unit of protein phosphatase 2A (Upstate Biotechnology, Inc., Lake Placid, NY) at 30 °C for 60 min in a solution containing 50 mM Tris-HCl (pH 7.5), 5 mM MgCl2, and 1 mM DTT. The reaction was terminated by the addition of SDS-PAGE sample buffer, and the samples were then subjected to SDS-7.5% PAGE, followed by protein staining.

Miscellaneous—Protein concentration was estimated by staining with Coomassie Brilliant Blue (Bio-Rad) using bovine serum albumin or rabbit skeletal myosin (for Dictyostelium myosin II) as a standard (26).

RESULTS

Unlike vertebrate MLCKs, Dictyostelium MLCK-A is not activated by Ca2+/CaM (13). Mutation of Thr166 to Glu in MLCK-A results in a 12-fold increase in its catalytic activity compared with the wild-type enzyme, suggesting that MLCK-A is likely to be activated through phosphorylation at Thr166 by an upstream protein kinase in Dictyostelium (17). This is also consistent with the finding of a previous study that T166A and T289A mutations abolish the increase in 32P incorporation into MLCK-A in metabolically labeled cells after stimulation with concanavalin A, which activates MLCK-A (17). However, there is no direct evidence of MLCK-A activation by Thr166 phosphorylation because a MLCK-A kinase capable of phosphorylating Thr166 has not been identified in Dictyostelium. Therefore, the activation mechanism of MLCK-A remains unclear. A search for metazoan homologs of Dictyostelium MLCK-A in the cDNA data base revealed that the catalytic domain of MLCK-A is most similar (~50% identical) to mammalian and Caenorhabditis elegans CaM-KI (Fig. 1) (27, 28). It has been demonstrated that both CaM-KI enzymes are phosphorylated by an upstream protein kinase (CaM-KK) at a Thr residue in their activation loop (Thr177 in human CaM-KI and Thr179 in C. elegans CaM-KI) that is equivalent to Thr166 in MLCK-A, resulting in a large increase in their catalytic efficiencies (21, 27, 28). We therefore investigated whether MLCK-A can be phosphorylated at Thr166 by CaM-KK, resulting in activation. If this is the case, the precise activation mechanism of MLCK-A could be determined using the surrogate MLCK-A kinase.

Phosphorylation and Activation of Dictyostelium MLCK-A by CaM-KKc—To assay for phosphorylation and activation of MLCK-A by CaM-KK, we used a minimum catalytic domain mutant of rat CaM-KKα (GST-CaM-KK-(84–434); CaM-KKc) lacking the N-terminal 83 amino acid residues and lacking a C-terminal regulatory region (residues 435–505) that includes an autoinhibitory domain and Ca2+/CaM-binding segments because the mutant has been shown to be constitutively active (29, 30). As shown in Fig. 2A, when we incubated recombinant GST-fused MLCK-A with CaM-KKc in the presence of MgATP and EGTA, robust phosphorylation was observed, whereas autophosphorylation of MLCK-A itself was very weak. Approximately 3.2 mol of phosphate was incorporated into MLCK-A within 120 min upon incubation with CaM-KKc. We measured MLCK-A activity under the same conditions using a peptide substrate corresponding to chicken gizzard MRLC (Kemptide) (Fig. 2B). The activity of recombinant MLCK-A was very low or undetectable under these conditions (1.5 μg/ml MLCK-A) and was not altered by incubation with MgATP in the absence of CaM-KKc. However, when we used a high concentration of the enzyme (20 μg/ml MLCK-A), we could detect wild-type MLCK-A activity (4.0 nmol/min/mg), as shown in Fig. 5B. In contrast, when MLCK-A was incubated with CaM-KKc in the presence of MgATP under the same conditions, MLCK-A was fully activated (1.3 μmol/min/mg) within 30 min (Fig. 2B) in association with incorporation of ~1 mol of phosphate into
matically enhanced by phosphorylation with CaM-KKc (0.23 
μmol/min/mg), resulting in efficient and stoichiometric phos-
phorylation of MRLC (−0.8 mol of phosphate/1 mol of MRLC).
We also determined the specificity constant ($k_{\text{cat}}/K_m$) by meas-
uring the phosphorylation rate at several subsaturating myo-
sin concentrations (0.2–1 mg/ml) as described previously (17).
The protein kinase activity of unactivated MLCK-A for 18-kDa
Dictyostelium MRLC was very low ($k_{\text{cat}}/K_m = 350 \text{ s}^{-1}$).
This is slightly lower that the value measured previously (670 
$\text{ s}^{-1}$) (17), most likely because of the GST tag present at the 
N terminus of the enzymes used in this study. For CaM-KKc-
treated MLCK-A, the activity was −140-fold higher ($k_{\text{cat}}/K_m = 
49.5/00 \text{ s}^{-1}$).

Identification of Phosphorylation Sites in Dictyostelium
MLCK-A—Although it has been demonstrated that recombi-
nant MLCK-A undergoes autophosphorylation at Thr$^{289}$ (14, 
17), we did not detect stoichiometric autophosphorylation of
MLCK-A in this present study (Fig. 2A). We therefore at-
tempts to identify the phosphorylation sites in both unacti-
vated and activated MLCK-A by mass spectrometry. When we
analyzed our recombinant unactivated MLCK-A by chymotryp-
sin digestion, followed by mass spectrometry analysis, we ob-
tained peptide sequences that covered 84% of the total amino 
acid sequence of MLCK-A (data not shown). Among them, we
detected a single phosphopeptide corresponding to residues
283–295 in MLCK-A (peptide A-1 in Table I). MS/MS analysis
revealed a single phosphorylation site at Thr$^{289}$ in the peptide
(Fig. 3A), indicating that MLCK-A was already autophospho-
rilated at Thr$^{289}$ in E. coli, which is consistent with a previous 
report (17). Therefore, we could not detect stoichiometric auto-
phosphorylation at Thr$^{289}$ in MLCK-A. In addition, we found
that wild-type MLCK-A migrated on SDS-7.5% polyacrylamide
gel slower than the protein phosphatase 2A-treated enzyme,
whereas the mobility of the T289A mutant on SDS-polyacryl-
amide gel was not altered by the phosphatase treatment (Fig.
4C), indicating that Thr$^{289}$ in wild-type MLCK-A was appar-
tently fully autophosphorylated. We also thought that activa-
tion of MLCK-A by CaM-KKc was due to phosphorylation at 
Thr$^{166}$ by CaM-KKc. Indeed, mass spectrometry analysis of
MLCK-A incubated with CaM-KKc in the presence of MgATP
for 120 min revealed that Thr$^{166}$ was phosphorylated in 
MLCK-A (peptide B-4 in Fig. 3B and Table I). This was also
confirmed by the finding that CaM-KKc-induced P$_i$ incorp-
oration into MLCK-A and activation of MLCK-A were completely 
abolished by the T166A mutation (Fig. 4, A and B). Although
we observed residual autophosphorylation of the T166A mu-
tant in either the presence or absence of CaM-KKc (Fig. 4A),
this was likely autophosphorylation at Thr$^{289}$ because P$_i$
incorporation was not observed with the T166A/T289A double 
mutant. Based on the mass spectrometry analysis, we also ob-
terved that MLCK-A (1–277), lacking Thr$^{289}$, possessed a 
single phosphorylation site at Thr$^{166}$ after 30 min of incubation
with CaM-KKc (data not shown). In addition to autophospho-
rylation sites at Thr$^{289}$ and phospho-Thr$^{166}$, we detected four 
other phosphorylation sites (Ser$^{42}$, Ser$^{155}$, Ser$^{251}$, and Ser$^{170}$) 
in activated MLCK-A (Table I). Because CaM-KKc could phos-
phorylate only Thr$^{166}$ in MLCK-A (Fig. 4A), these Ser residues 
are likely autophosphorylation sites. Autophosphorylation at 
multiple Ser residues of MLCK-A subsequent to activation is 
consistent with the results shown in Fig. 2A. When we ana-
lized the phosphorylation sites in the T166E mutant, which 
has been shown to be a phosphorylation-mimicking mutant 
(17), we detected two phosphorylation sites at Ser$^{251}$ and Ser$^{170}$
in addition to phospho-Thr$^{289}$ by mass spectrometry, but we
could not observe phosphorylation at Ser$^{42}$ and Ser$^{155}$ in the 
mutant (data not shown). This may indicate that autophospho-

![Image](image-url)
rylation at Ser$^{42}$ and Ser$^{155}$ occurs only in vitro and that the activity of the T166E mutant is not enough to autophosphorylate these Ser residues (Ser$^{42}$ and Ser$^{155}$) compared with the enzyme phosphorylated at Thr$^{166}$ (Table I). However, this autophosphorylation subsequent to phosphorylation at Thr$^{166}$ was slow and did not appear to be involved in the enzymatic regulation in MLCK-A (Fig. 2B), suggesting that it may not be physiological. In addition, only Thr phosphorylation of MLCK-A has been observed in vivo (17).

Taken together, these results indicate that CaM-KKc is capable of activating MLCK-A through phosphorylation at Thr$^{166}$. This is consistent with the finding that maximum activation of MLCK-A by CaM-KKc was achieved within 30 min and that, at this time, 1 mol of phosphate was incorporated into the enzyme (Fig. 2, A and B). Notably, the maximum activity of activated MLCK-A (1.3 μmol/min/mg) was 300-fold higher than that of the unactivated enzyme under our conditions, indicating that mutation of Thr$^{166}$ to Glu only partially (12-fold higher compared with the wild-type enzyme) mimicked the effect of phosphorylation on the activity (17). Also the activity measured for CaM-KKc-treated MLCK-A is comparable with that measured in crude Dictyostelium lysate containing active MLCK-A (16). Therefore, we could address the activation mechanism of MLCK-A using this surrogate MLCK-A kinase, CaM-KKc.

**Requirement of Thr$^{289}$ Autophosphorylation for Efficient Activation of Dictyostelium MLCK-A**—First, we examined the role of autophosphorylation at Thr$^{289}$ in activation of MLCK-A (Fig. 4). P$_i$ incorporation into the T289A mutant was significantly reduced (−20%) compared with the wild-type enzyme when the mutant was incubated with CaM-KKc in the presence of MgATP for 30 min (Fig. 4A). In addition, the phosphorylation-mimicking mutant T289E was more efficiently phosphorylated by CaM-KKc compared with the T289A mutant within 30 min, but the mutant was less efficiently phosphorylated by CaM-KKc compared with the wild-type enzyme under these conditions, indicating that the Glu mutation partially mimicked the effect of phosphorylation at Thr$^{289}$. We therefore performed a kinetic experiment to examine activation of the Thr$^{289}$ mutants (Fig. 4D). As shown in Figs. 2B and 4D, wild-type MLCK-A, which was autophosphorylated at Thr$^{289}$, was rapidly activated by CaM-KKc ($t_{1/2} \sim 8\text{ min}$). In contrast, the T289A mutant was activated very slowly ($t_{1/2} > 90\text{ min}$), and activation of T289E was more rapid ($t_{1/2} \sim 20\text{ min}$) than that of the T166A mutant, which is consistent with the P$_i$ incorporation into these MLCK-A mutants induced by CaM-KKc (Fig. 4A). These results indicate that autophosphorylation at Thr$^{289}$ is required for efficient phosphorylation at Thr$^{166}$ by an upstream kinase, which results in maximum activity.

**Identification of an Autoinhibitory Domain in Dictyostelium MLCK-A**—In the absence of phosphorylation at Thr$^{166}$ in MLCK-A, the activities of both wild-type MLCK-A and the T289A mutant were very low or below the detectable level under the conditions employed (1.5 μg/ml MLCK-A) (Figs. 2 and 4). A previous study demonstrated that a truncated form lacking the C-terminal 37 amino acid residues exhibits −10-fold higher activity compared with full-length MLCK-A, indicating the presence of an autoinhibitory domain in its C-terminal region (14). We wished to map this autoinhibitory domain and to determine the relationship between autoinhibition and activation of MLCK-A. A series of C-terminal truncation mutants were expressed (Fig. 5A), and we used a high concentration of enzyme (20 μg/ml) to accurately measure the activities of these enzymes prior to activation by CaM-KKc (Fig. 5B). Whereas truncation at residue 288 did not alter MLCK-A activity, truncation at residue 282 induced a significant increase in activity (−15-fold), and the activities of additional truncation mutants (MLCK-A(1–277) and MLCK-A(1–270)) were indistinguishable from that of MLCK-A(1–282) (Fig. 5B). This indicates that residues 283–288 function as an autoinhibitory region in MLCK-A to suppress MLCK-A activity. In contrast, phosphorylation and activation of all mutants by CaM-KKc were similar to those of the wild-type enzyme (Fig. 5C), suggesting that activation of MLCK-A by an upstream kinase is not affected by its autoinhibitory mechanism. Although the activities of the mutants lacking an autoinhibitory region (MLCK-A(1–282), MLCK-A(1–277), and MLCK-A(1–270)) were −15-fold higher than that of autoinhibited wild-type MLCK-A, they were −20-fold lower than that of the activated form under these conditions.

**Effect of Activation on the Kinetic Parameters of Dictyostelium MLCK-A**—It was important to determine how activation by an upstream kinase alters the kinetic parameters of MLCK-A. The high molecular mass of myosin (274 kDa) made it impossible to obtain saturating concentrations in our assays; so for this substrate, $k_{cat}/K_m$ could not be resolved into its components, $k_{cat}$ and $K_m$. We therefore determined the kinetic constants of wild-type MLCK-A (autophosphorylated at Thr$^{289}$), activated MLCK-A, and unactivated and activated MLCK-A(1–277) lacking an autoinhibitory region (MLCK-A(1–282), MLCK-A(1–277), and MLCK-A(1–270)) as $k_{cat}$ values (Table II). Deletion of the autoinhibitory region (MLCK-A(1–277)) increased the $k_{cat}$ value (−57-fold) compared with basal MLCK-A without significant effects on the affinities for both ATP and Kemptamide. The $k_{cat}$ value of MLCK-A(1–277) was further increased (−19-fold) by Thr$^{166}$ phosphorylation (Table II). Deletion of the autoinhibitory region (MLCK-A(1–277)) increased the $k_{cat}$ value (6.4 s$^{-1}$) of activated wild-type MLCK-A. When we used recombinant Dictyostelium MRLC as a substrate, the kinetic changes were similar to what we observed with Kemptamide, except that the catalytic efficiency ($k_{cat}/K_m$) of basal MLCK-A for MRLC was −8-fold higher than that for the peptide substrate. As a result, the $k_{cat}$ value of wild-type MLCK-A

### Table II

| MLCK-A                | $K_m$ for ATP (μM) | $k_{cat}$ (μM s$^{-1}$) | $K_m$ for Kemptamide (μM) | $k_{cat}$ (μM s$^{-1}$) | $K_m$ for MRLC (μM) | $k_{cat}$ (μM s$^{-1}$) |
|----------------------|-------------------|-------------------------|---------------------------|-------------------------|-------------------|------------------------|
| WT MLCK-A (autophosphorylated at Thr$^{289}$) | 31               | 0.008                   | 22                        | 0.065                   | 3                  | 15.3                   |
| Activated WT MLCK-A  | 51               | 0.46                    | 12                        | 7.1                     | 23                | 0.99                   |
| MLCK-A(1–277)        | 49               | 0.46                    | 23                        | 0.99                    | 116               | 15.3                   |
| Activated MLCK-A(1–277) | 57           | 8.7                     | 17                        | 15.3                    |                   |                        |
was increased ~100-fold by activation without a significant decrease in the $K_m$ for MRLC. Similar to what we observed with the peptide substrate, the $k_{cat}$ value of MLCK-A (1–277) was further increased (~15-fold) by Thr$^{166}$ phosphorylation without any effects on the affinity for MRLC. These results suggest that phosphorylation at Thr$^{166}$ is accompanied by a large increase in the catalytic efficiency of MLCK-A, which is associated with suppression of the autoinhibitory mechanism and an increased $k_{cat}$ value. The autoinhibitory region (residues 283–288) plays a role in the complete suppression of MLCK-A activity in the absence of activation; however, releasing the autoinhibitory region from the catalytic core is not enough to generate maximum MLCK-A activity.

**DISCUSSION**

The activation mechanism of MLCK-A studied in this paper is somewhat analogous to that of the CaM kinase cascade, which is composed of an upstream CaM-KK and downstream CaM-KI and CaM-KIV. CaM-KK phosphorolysates a specific Thr residue (Thr$^{177}$ in CaM-KI and Thr$^{196}$ in CaM-KIV) in each of their activation loops, resulting in the remarkable activation of both downstream CaM-Ks in the presence of Ca$^{2+}$/CaM. Previous studies demonstrated that CaM-KKc is unable to phosphorylate and activate CaM-KI and CaM-KIV in the absence of Ca$^{2+}$/CaM because phosphorylation in the activation loop is blocked by the autoinhibitory regions of CaM-KI and CaM-KIV (29, 30). Thus, both downstream CaM-Ks require release of autoinhibitory regions from their catalytic domains through Ca$^{2+}$/CaM binding to their regulatory domains to expose the activation Thr residue to CaM-KK (22, 27, 29). In contrast, MLCK-A does not seem to require either cofactors or release of autoinhibition for subsequent phosphorylation at Thr$^{166}$ by an upstream kinase, but it does require Thr$^{289}$ autophosphorylation for efficient phosphorylation at Thr$^{166}$ by an upstream kinase (Fig. 4). Unlike CaM-KI phosphorylated at Thr$^{177}$, whose activity is completely suppressed by the autoinhibitory region in the absence of Ca$^{2+}$/CaM, the activity of MLCK-A phosphorylated at Thr$^{166}$ is no longer suppressed by its autoinhibitory region (residues 283–288). This is similar to the fact that CaM-KIV generates a significant degree of the autonomous activity through phosphorylation at Thr$^{196}$ by CaM-KK (31). However, the mechanism of generation of the Ca$^{2+}$/CaM-independent activity of activated CaM-KIV has remained unclear. It is intriguing that MLCK-A undergoes autophosphorylation only at Thr$^{289}$ in the absence of activation, whereas the enzyme is maintained in an autoinhibited state by the interaction of the autoinhibitory region (residues 283–288) with the catalytic core. Autophosphorylation at Thr$^{289}$ has been shown to be an intramolecular event (13), which could be explained by the likely localization of Thr$^{289}$ in or close to the catalytic cleft by interaction of the adjacent autoinhibitory segment (residues 283–288) with the catalytic domain. It then follows that autophosphorylation at Thr$^{289}$ might induce a conformational change in the MLCK-A regulatory region that fully exposes Thr$^{166}$ in the catalytic domain to an upstream kinase. This is supported by the fact that mutation of Thr$^{289}$ to non-phosphorylatable Ala significantly reduced the efficiency of phosphorylation at Thr$^{166}$ by CaM-KKc. Notably, autophosphorylation at Thr$^{289}$ did not in itself significantly suppress the autoinhibitory function to generate MLCK-A activity compared with the enhanced activity of MLCK-A mutants lacking an autoinhibitory region (Fig. 5B). Taken together, these results show that, once MLCK-A was phosphorylated at Thr$^{166}$ by an upstream kinase, MLCK-A exhibited a maximum level of kinase activity by releasing the autoinhibitory region from the catalytic core as well as by increasing the efficiency of catalysis. Because we examined the activation mechanism of MLCK-A, including the role of Thr$^{289}$ autophosphorylation and the effect of Thr$^{166}$ phosphorylation in vitro, activation of the enzyme in intact Dictyostelium remains to be examined.

It has been shown that the addition of cGMP to crude lysates from vegetative and developing cells (but not from starved cells lacking MLCK-A) induces MRLC phosphorylation in vitro, indicating that cGMP stimulates MLCK-A activity (16). However, cGMP itself is not capable of directly activating MLCK-A (16), indicating two possibilities for the activation mechanism of MLCK-A: 1) MLCK-A kinase is directly regulated by cGMP signaling; and/or 2) MLCK-A is regulated by a cGMP-regulated protein to expose Thr$^{166}$ to MLCK-A kinase, analogous to the activation mechanism of the CaM kinase cascade, in which both the upstream and downstream kinases are tightly regulated by Ca$^{2+}$/CaM to constitute a signaling pathway. Although MLCK-A kinase in Dictyostelium remains to be identified, our present results suggest that the second explanation is quite unlikely because MLCK-A does not require any cofactors or regulators for phosphorylation at Thr$^{166}$ and activation by a constitutively active upstream kinase. Therefore, the upstream MLCK-A kinase activity is likely to be either directly or indirectly controlled by cGMP signaling. However, we cannot rule out the possibility that the regulatory protein(s) may bind to MLCK-A to modulate the efficiency of Thr$^{166}$ phosphorylation. Interestingly, recent studies have identified at least four candidate cGMP-binding targets in Dictyostelium (32), and cells with a deletion of the two cGMP-binding targets GbpC and GbpD show a reduced increase in MRLC phosphorylation in response to chemoattractants, suggesting a possible involvement of cGMP-binding proteins in chemotaxis-induced MRLC phosphorylation in Dictyostelium (33). Our study raises the possibility that the MLCK-A kinase could be structurally related to CaM-KK, especially with respect to its catalytic domain, which might be regulated by cGMP signaling. Another possibility is that a CaM-KK homolog that is also a Ca$^{2+}$/CaM-dependent enzyme may exist in Dictyostelium and render MLCK-A Ca$^{2+}$-dependent, as in the case of Ca$^{2+}$/CaM-regulated vertebrate MLCK, because a CaM-KK homolog has been identified in lower eukaryotes such as *C. elegans* (34) and *Aspergillus nidulans* (35). Therefore, further studies on the identification and characterization of the MLCK-A kinase will be required to clarify the detailed mechanism of the MLCK-A activation cascade and its physiological significance during cytokinesis and chemotaxis in Dictyostelium.

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