Boundary of the Autoinhibitory Region of Smooth Muscle Myosin Light-Chain Kinase†

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ABSTRACT: It has been proposed that myosin light-chain kinase (MLCK) activity is inhibited in the absence of Ca^{2+}/calmodulin by a pseudosubstrate sequence [Kemp, B. E., Pearson, R. B., Guerriero, V. J., Bagchi, I., & Means, A. R. (1987) *J. Biol. Chem. 262,* 2542–2548]. To evaluate this hypothesis, the role of a cluster of basic residues, Arg797–Arg798–Lys799, which are essential for the pseudosubstrate sequence, in the inhibition of MLCK was studied. A full-length cDNA of chicken gizzard MLCK was obtained, and the recombinant MLCK which contains the entire amino acid sequence was expressed in *Escherichia coli.* The Ca^{2+}/calmodulin-dependent activity of the recombinant MLCK was comparable to that of the naturally isolated MLCK. Two truncation mutants, MT799 and MT796, were produced, of which MT799 but not MT796 contained a cluster of basic residues. Neither MT799 nor MT796 bound calmodulin, and kinase activity was inhibited (similar to MLCK activity in the absence of Ca^{2+}/calmodulin). However, the kinase activity of the mutants was increased markedly by subsequent tryptic proteolysis. The tryptic digestion of the mutants initially produced a 64-kDa fragment then, subsequently, the 61-kDa fragment, and the increase in activity coincided with the appearance of the 61-kDa fragment. This was similar to the digestion profile of native MLCK, and it is known that the 61-kDa fragment is the constitutively active kinase [Ikebe, M., Stepinska, M., Kemp, B. E., Means, A. R., & Hartshorne, D. J. (1987) *J. Biol. Chem. 262,* 13828–13834]. The results show that the Arg797–Arg798–Lys799 residues are not essential for the inhibition of MLCK, suggesting that the critical region of the autoinhibitory domain is on the N-terminal side of Ala796. Therefore, the pseudosubstrate inhibitor concept of MLCK may require reconsideration.

Myosin light-chain kinase (MLCK)[1] is a Ca^{2+}/calmodulin-dependent protein kinase which specifically phosphorylates the regulatory light chain of myosin. In smooth muscle, this is a key regulatory enzyme for the activation of contractile machinery (Hartshorne, 1987; Sellers & Adelstein, 1987; Kamm & Stull, 1989). The mechanism by which calmodulin binding can activate MLCK has been studied using protein biochemical and molecular biological techniques. For smooth muscle MLCK, the catalytic domain occupies the central portion of the molecule, as determined by the sequence homology noted to be conserved among various protein kinases (Olson et al., 1990; Gallagher et al., 1991). Consistent with this, an active proteolytic fragment from this region (residues 287–776) of smooth muscle MLCK is produced, following limited tryptic digestion (Ikebe et al., 1987, 1989).

The calmodulin-binding site of smooth muscle MLCK was first demonstrated by isolation of the peptide phosphorylated by cAMP-dependent protein kinase (Lukas et al., 1986). It was known that cAMP-dependent protein kinase phosphorylates smooth muscle MLCK and that the phosphorylation reduces the affinity of MLCK for calmodulin (Adelstein et al., 1978), suggesting that the phosphorylation site is in close proximity to the calmodulin-binding region. The peptide (797–816) that contained the phosphorylation site showed strong affinity (K_d = 1 nM) toward calmodulin (Lukas et al., 1986). The residues important for the binding to calmodulin were also studied by truncation or substitution of the amino acid residues at the calmodulin-binding site of the recombinant MLCK (Bagchi et al., 1989). The amino acid residues essential for calmodulin binding were recently determined by analyzing the three-dimensional structure of the MLCK peptide–calmodulin complex with multidimensional NMR spectroscopy (Ikura et al., 1992; Meador et al., 1992). It was found that two hydrophobic residues (Trp800 and Leu813) play an essential role in the binding of calmodulin.

The mechanism of calmodulin-mediated MLCK activation has been investigated. Of interest is that an autoinhibitory region was found at the N-terminal side of the calmodulin-binding site. The synthetic peptides modeled after the N-terminal side of the calmodulin-binding region of MLCK showed potent inhibitory activity against native MLCK (Kemp et al., 1987) as well as constitutively active MLCK (Ikebe et al., 1987). This suggested that the sequence residing at the N-terminal side of the calmodulin-binding site may function as an intramolecular inhibitor. Furthermore, it was also shown (Ikebe et al., 1987) that the proteolysis of smooth muscle MLCK yields a 64-kDa inactive fragment, which is further proteolyzed to produce a 61-kDa constitutively active kinase fragment. This also indicated the existence of the autoinhibitory region toward the N-terminus of the calmodulin-binding site.

Kemp et al. (1987) thus proposed that inhibition for smooth muscle MLCK is due to the interaction of the catalytic site

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[1] Abbreviations: MLCK, myosin light-chain kinase; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; DTT, dithiothreitol; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; LB, Luria–Bertani medium; IPTG, isopropyl 1-β-D-thiogalactopyranoside; LC_{20}, 20 000-Da light chain of smooth muscle myosin; PMSF, phenylmethanesulfonyl fluoride.

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with a pseudosubstrate sequence of MLCK at the inhibitory region. This pseudosubstrate inhibitor hypothesis is based upon the sequence similarities between the myosin regulatory light-chain sequence at the N-terminal side of serine 19 (phosphorylation site by MLCK) and the MLCK sequence at the N-terminal side of the calmodulin-binding region. Alignment of His805 of MLCK with Ser19 of the regulatory light chain of myosin resulted in the finding that several basic residues of MLCK match the basic residues of the light chain. Among them, a cluster of basic residues of MLCK (Arg797–Arg798–Lys799) was suggested to play an important role as a pseudosubstrate inhibitor (Kemp et al., 1987; Pearson et al., 1988, 1991). Recently, the structure of the smooth muscle MLCK catalytic core was modeled by using the crystallographic coordinates of the cAMP-dependent protein kinase catalytic subunit and the bound inhibitor peptide (Knighton et al., 1992), and the possible interaction between the basic residues in the pseudosubstrate sequence and the acidic residues in the catalytic domain was suggested.

The boundary of the autoinhibitory region has been studied by determining the C-terminal end of the inactive fragments of MLCK produced by various proteases (Kemp et al., 1987; Pearson et al., 1988, 1991). The results are controversial. Pearson et al. reported (1988) that the inactive fragment contains the entire pseudosubstrate sequence, while the results reported by Ikebe et al. (1989) suggest that the inactive fragment of MLCK lacks the important portion of the pseudosubstrate sequence.

In the present study, we produced mutant smooth muscle MLCK and evaluated the importance of the RRK motif, which is thought to be essential for the pseudosubstrate sequence.

MATERIALS AND METHODS

Protein Preparation and Protein Biochemical Procedure. MLCK was prepared from frozen turkey gizzard (Ikebe et al., 1987), and calmodulin was prepared from bull testes (Walsh et al., 1983). The 20 000-Da light chain of myosin was prepared from myosin as described previously (Ikebe et al., 1988). MLCK activity was measured at 25 °C using isolated 20 000-Da light chains of smooth muscle myosin (0.1 mg/mL) as the substrate in 30 mM Tris-HCl (pH 7.5), 4 mM $MgCl_2$, 0.2 mM $CaCl_2$, 5 μg/mL calmodulin, and 0.1 mM [γ-^{32}P]ATP. To measure Ca^{2+}/calmodulin-independent activity, 1 mM EGTA was added to the reaction mixture instead of 0.2 mM $CaCl_2$ and 5 μg/mL calmodulin.

SDS–PAGE was carried out on 7.5–20% polyacrylamide gradient slab gels by using the discontinuous buffer system of Laemmli (1970). Molecular masses were estimated using known molecular mass markers of smooth muscle myosin heavy chain (200 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), myosin regulatory light chain (20 kDa), and α-lactalbumin (14.2 kDa).

MLCK activity was determined as described by Walsh et al. (1983) using the isolated 20-kDa light chain of myosin as a substrate. For MLCK mutants, the fractions were digested with 20 μg/mL trypsin (TPCK treated, Worthington) in buffer containing 30 mM Tris-HCl (pH 7.5), 1 mM EGTA, and 30 mM NaCl at 25 °C for 10 min, and the reaction was terminated with 30 μg/mL soybean trypsin inhibitor (Sigma). The fractions were then subjected to MLCK activity assay.

The concentration of MLCK in partially purified MLCK mutants was estimated from densitometric analysis (Hoefer Scientific Instruments GS300) of the Western blot stained by the enhanced chemiluminescence detection method. Various concentrations of the purified natural MLCK were also subjected to the densitometric analysis and used as a standard.

Western blotting was carried out as described previously (Higashihara et al., 1989) with slight modifications. The protein-transferred Immobilon P sheets (Millipore) were impregnated in TBS buffer (50 mM Tris-HCl (pH 7.5) and 0.15 M NaCl) containing 5% nonfat milk for 1 h at room temperature to saturate nonspecific protein-binding sites. The monoclonal antibody of gizzard MLCK, termed LKH7 (Araki & Ikebe, 1991), was used as the first antibody. The sheets were incubated with the first antibody in 5% milk–TBS overnight at 4 °C and then washed with TBS four times. Horseradish peroxidase conjugated antimouse IgG antibodies (Bio-Rad) were used as the second antibody. The sheets were incubated with the second antibody in 5% milk–TBS, incubated at room temperature for 1 h, and then washed with TBS five times. The sheets were stained using the enhanced chemiluminescence detection method (Amersham).

Construction of the Wild-Type MLCK Expression Vector. Chicken gizzard λ ZAP cDNA library was screened with oligonucleotides complementary to gizzard MLCK cDNA (Guerriero et al., 1986). Forty positive clones were obtained, and these clones were in vivo excised into the pBluescript SK(–) vector. One of them, pML-3, had an insert length of 3 kb. The sequence of this clone was determined by the dideoxynucleotide method (Sanger et al., 1977) and compared with the published chicken gizzard MLCK cDNA sequence. It was found that the clone, pML-3, did not contain the first 150 base pairs of the coding region. Therefore, total RNA was prepared from chicken gizzard and used as a template for the reverse transcriptase coupled polymerase chain reaction to produce the cDNA fragment including the first 150 nucleotides (Figure 1).

Two oligonucleotides were made and used as the primers for the polymerase chain reaction (PCR). One was 5′-CGGAATTCAAATGGACTTCGAGCAAACC-3′, containing an *Eco*RI site at the 5′-side, and the other was 5′-CACCATCTGCTACTTTAGCATC-3′. The PCR product contained an *Eco*RI site at the 5′-terminus and a *Hin*dIII site at the 3′-side. The DNA fragment was digested with *Eco*RI and *Hin*dIII, subcloned into pBluescriptII SK(+) (Stratagene), and termed pML-5. The insertion was confirmed by sequencing and was found to consist of a sequence identical to the 5′-region of the published gizzard MLCK cDNA (Olson et al., 1990). pML-3 was digested with *Hin*dIII (unique *Hin*dIII site in MLCK cDNA and a *Hin*dIII site in the multicloning site of the vector pBluescript(II) SK(–)), and the cDNA fragment was purified by agarose gel electrophoresis followed by extraction using GENECLEAN (BIO101). This DNA fragment was subcloned in frame to the *Hin*dIII site of pML-5 (Figure 1). The generated plasmid, pBML, has the full-length MLCK cDNA. The full-length MLCK cDNA was then excised by *Eco*RI digestion and in frame inserted into the pT7-7 expression vector (kindly supplied by Dr. Stanley Tabor), which contains the T7 promoter and a Shine–Dalgarno sequence. The transformants were selected and analyzed by restriction enzyme digestion for proper orientation. The construct is termed pTML3 (Figure 1).

Site-Directed Mutagenesis. A transformer Site-Directed Mutagenesis Kit (Clontech Inc.) was used for the mutation. Complementary oligonucleotides with mismatches were synthesized to serve as primers: primer T-1,5′-GCCAGAAGAAAATGATAGAAAACAGGCCATG-3′; primer T-2,5′-GAAGAATATATGGCCTGATGAAAATGGCAGAAAAC-3′. T-1 creates two termination codons after Lys799,

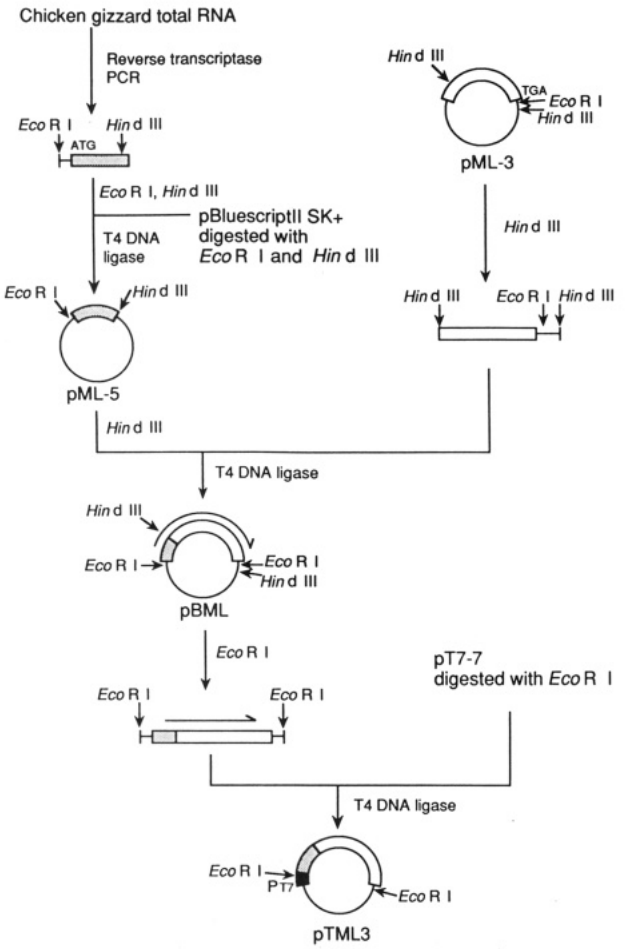

FIGURE 1: Construction of the wild-type MLCK expression vector. A detailed strategy for constructing the expression vector is described in Materials and Methods. The white box in pML-3 represents a partial MLCK cDNA which does not contain the first 150 nucleotides of the cDNA. The first 450 base pairs of the 5′-terminus were synthesized by reverse transcription and PCR from chicken gizzard total RNA. The cDNA fragment is represented as a shaded box. ATG on the shaded box and TGA on the white box indicate initiation and termination codons, respectively. Arrows on the pBML and on the *Eco*RI fragment derived from pBML indicate the transcriptional direction, PT7, and T7 promoter.

and T-2 creates two after Ala796. Primer R-1, 5′-CTTTAAGAAGGAGATATACGTATGGCTAGAATTCAAATG-3′, was also made to eliminate the unique *Nde*I site, which is located at the initiation codon in the MLCK expression vector pTML3. [One nucleotide was changed to eliminate the *Nde*I site without changing the amino acid residue (Figure 2).] Two pairs of primers,(T-1,R-1) and (T-2,R-1), were used to make truncated mutants MT799 and MT796, respectively (Figure 2).

Two oligonucleotide primers were annealed to one strand of denatured, double-stranded template, pTML3. DNA was then synthesized using T4 polymerase and ligated by T4 DNA ligase. T4 DNA polymerase was used to elongate the primers because this polymerase lacks strand displacement activity, preventing any annealed primers from being displaced during polymerization. The mixture of mutated and unmutated DNAs was transformed with BMH 71-18 mut S *Escherichia coli* strain defective in mismatch repair. Transformants were grown in LB medium containing 50 μg/mL ampicillin overnight at 37 °C with continuous shaking. DNA was prepared from the mixed bacterial population and then subjected to *Nde*I digestion. Since the mutated DNA lacks the unique *Nde*I site, it is resistant to digestion with *Nde*I

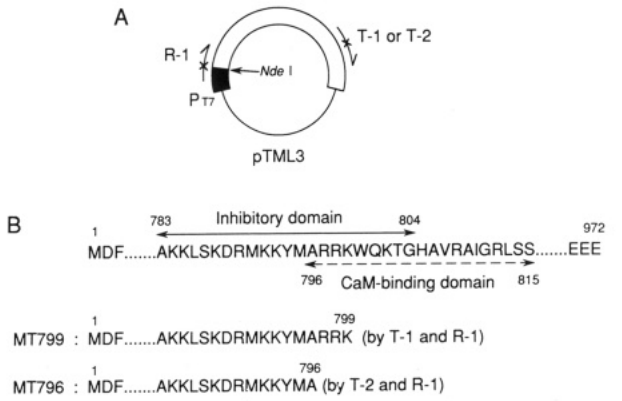

FIGURE 2: Site-directed mutagenesis for making truncated MLCK mutants. The mutation scheme was as described in Materials and Methods. Three primers were used to mutate the wild-type MLCK expression vector, pTML3 (A). Primers T-1 and T-2 create two termination codons each in the inhibitory domain. Primer R-1 was used to delete the *Nde*I site, which was unique in pTML3. After mutagenesis, parental plasmids were linearized by *Nde*I digestion, and only circular plasmids with the proper mutations were used for the transformation of *E. coli*. (B) Two truncated MLCK mutants generated with two pairs of primers.

while the original DNA is sensitive to digestion and is linearized, rendering it at least 100 times less efficient in the transformation of bacterial cells. The digested DNA was used to transform *E. coli* XL1-Blue. Single colonies on an LB plate with 50 μg/mL ampicillin were picked up and grown in LB with 50 μg/mL ampicillin overnight at 37 °C with continuous shaking. The plasmid DNAs were prepared and selected by *Nde*I digestion. The DNAs that were not digested by *Nde*I were subjected to sequence analysis to confirm the mutation.

Expression of Recombinant MLCK in E. coli. *E. coli* strain BL21 (DE3) was transformed with pTML3 or the plasmids with the mutagenized cDNAs of MLCK. This bacteria has the T7 RNA polymerase gene in its genome, which can be transcribed by the addition of isopropyl 1-β-D-thiogalactopyranoside (IPTG). The transformant was grown in LB medium with 50 μg/mL ampicillin. When the absorbance at 600 nm reached 0.5–0.7, IPTG was added to the culture (final concentration 1 mM). The cells were harvested 6 h after induction. Cells were collected by centrifugation at 4000*g* for 10 min. To examine the expression of recombinant MLCK, the packed cells were sonicated in 1% SDS, 25 mM Tris-HCl (pH 6.8), 15% glycerol, and 0.01% bromophenol blue (BPB) and then dissolved by boiling for 3 min and subjected to SDS–PAGE analysis.

The pellet was washed with 30 mM Tris-HCl (pH 7.5) and then sonicated in lysis buffer (30 mM Tris-HCl (pH 7.5), 1 mM DTT, 1 mM EDTA, 10 μg/mL leupeptin, 0.1 mM $CaCl_2$, and 3% Triton X-100). After sonication, the sample was centrifuged in 1.5-mL microtubes at 10000*g* for 15 min. For wild-type MLCK, the supernatant was filtered with a 0.45-μm filter (Millipore Corp.) and then subjected to calmodulin–Sepharose 4B column chromatography. The column was preequilibrated with 30 mM Tris-HCl (pH 7.5), 30 mM NaCl, 0.2 mM $CaCl_2$, and 1 mM DTT. The sample was loaded onto the column, and the column was washed with the equilibration buffer. The column was then washed with equilibration buffer plus 0.5 M NaCl to remove nonspecific binding proteins. Finally, MLCK was eluted with buffer containing 1 mM EGTA, 0.5 M NaCl, 1 mM DTT, and 30 mM Tris-HCl (pH 7.5).

For MT799 and MT796, the extract was loaded onto a DEAE–Sephacel column equilibrated with 15 mM Tris-HCl

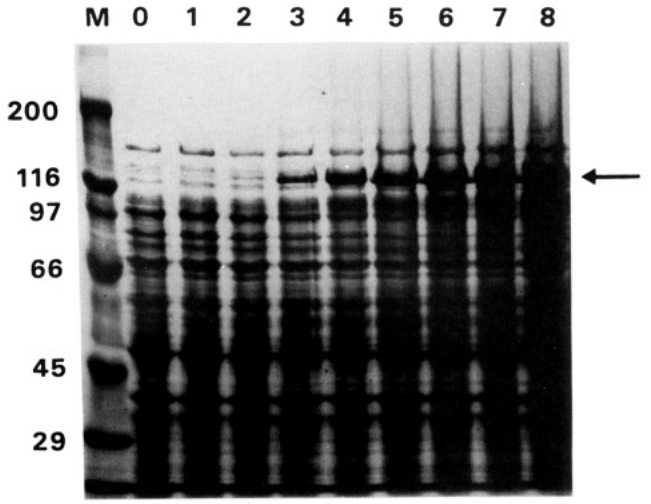

FIGURE 3: Time course of the expression of recombinant MLCK in *E. coli*. Expression of recombinant MLCK was induced by the addition of IPTG. The expression pattern was monitored by SDS–PAGE as described in Materials and Methods: lane M, molecular mass marker; lane 0, cell lysate just before induction; lanes 1–8, cell lysates 1–8 h after induction, respectively. The MLCK expressed is shown by an arrow. Molecular mass (kDa) is shown in the left margin.

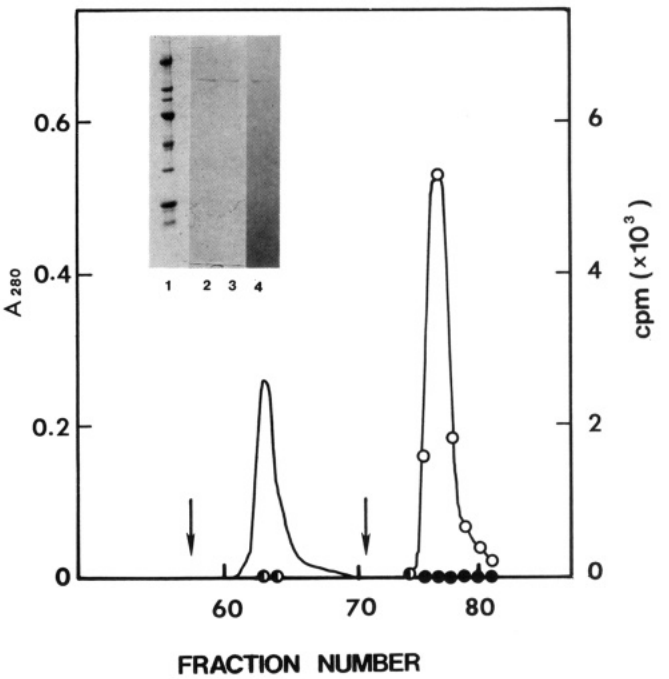

FIGURE 4: Purification of recombinant MLCK by calmodulin-affinity column chromatography. The recombinant MLCK was extracted as described in Materials and Methods and subjected to calmodulin–Sepharose 4B affinity chromatography. The crude extract was loaded onto the column and washed with equilibration buffer. The column was washed with equilibration buffer containing 0.5 M NaCl (indicated by an arrow), and then MLCK was eluted with buffer containing 1 mM EGTA (indicated by another arrow). The fractions were subjected to MLCK activity assay (○, ●). The activity is expressed by the radioactivity of ^{32}P incorporated into the light chain with reaction time of 2 min: ○, activity in the presence of Ca^{2+}/calmodulin; ●, activity in the absence of Ca^{2+}/calmodulin. The inset shows the purified recombinant MLCK: lane 1, molecular mass standard; lane 2, fraction 77; lane 3, fraction 78; lane 4, Western blot of purified recombinant MLCK. A_{280} is shown by the solid line.

(pH 8.3), 1 mM EGTA, 1 mM DTT, and 0.1 mM PMSF. The mutant MLCK was eluted by a linear NaCl gradient (0–0.5 M). The fractions containing MLCK were dialyzed against 30 mM NaCl, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, and 10 mM KP_i (pH 7.5) (buffer A). The fraction was applied to a hydroxylappatite column equilibrated with buffer A. The column was washed with buffer A plus 0.5 M NaCl, and then MLCK was eluted by a linear gradient of KP_i (0–0.5 M). The fractions were assayed for MLCK activity and used as partially purified MLCK mutants.

RESULTS

E. coli strain BL21 (DE3) was transformed with the wild-type MLCK construct, pTML3 (Figure 1), and the recombinant wild-type MLCK was expressed by the addition of IPTG. Figure 3 shows the SDS–PAGE patterns of the cell lysate at various times after IPTG induction.

A peptide band with an apparent molecular mass of 130 kDa appeared at 3 h after induction by IPTG. The expression of the 130-kDa peptide increased with time after IPTG induction and reached a maximum level at 6 h (Figure 3). The 130-kDa peptide was recognized by the LKH7 anti-gizzard MLCK antibody, which confirmed that the 130-kDa peptide was recombinant MLCK (Figure 6).

The wild-type MLCK was extracted from packed cells and purified by calmodulin–Sepharose 4B chromatography (Figure 4) (see Materials and Methods). The 130-kDa peptide was eluted from the column with EGTA-containing buffer. The fractions containing this peptide exhibited Ca^{2+}/calmodulin-dependent kinase activity against the 20-kDa light chain of myosin. Furthermore, the 130-kDa peptide reacted with LKH7, as judged by Western blotting analysis (Figure 4, inset); therefore, it was concluded that the 130-kDa peptide was the recombinant MLCK expressed in *E. coli*. The activity was completely Ca^{2+}/calmodulin-dependent, and the specific activity was comparable to that of naturally isolated MLCK from chicken gizzard (Table I).

E. coli cell BL21 (DE3) was also transformed with the truncated MLCK constructs, MT799 and MT796. The expression of the truncated mutants of MLCK was induced by IPTG. Figure 5 shows the SDS–PAGE patterns of cell lysate before and after the addition of IPTG. For both MT799 and MT796 constructs, a single polypeptide of apparent molecular mass of 100 kDa appeared after IPTG induction (Figure 5). Western blotting analysis revealed that the 100-kDa peptide is the truncated MLCK (Figure 6). The apparent molecular mass of 100 kDa was also consistent with the number of amino acid residues in the truncated mutants (799 and 796 residues for MT799 and MT796, respectively), that is, the native MLCK consisting of 972 residues has an apparent molecular mass of 130 kDa as estimated by SDS–PAGE.

Table I: Specific Activities of the Recombinant MLCKs[a]

	undigested		tryptic digested	
	EGTA	Ca^{2+}/calmodulin	EGTA	Ca^{2+}/calmodulin
MLCK (natural)	0.017	1.17	1.15	0.98
rMLCK (wild type)	0.019	2.69	1.37	1.07
MT799	0.042	0.072	2.36	1.31
MT796	0.048	0.048	1.63	0.95

[a] The activities (μmol/min·mg) are determined in the presence of 30 mM NaCl, 4 mM $MgCl_2$, 100 μM [γ-^{32}P]ATP, 0.2 mg/mL LC_{20}, 30 mM Tris-HCl (pH 7.5), and 1 mM EGTA or 0.2 mM $CaCl_2$/5 μg/mL calmodulin at 25 °C.

The truncated mutants of MLCK were partially purified by a series of liquid chromatography elutions (see Materials and Methods). Figure 7 shows the elution profile of hydroxylappatite chromatography for the purification of MT799 and MT796. Both were eluted during the application of the KP_i gradient. The elution of the kinase was also confirmed by

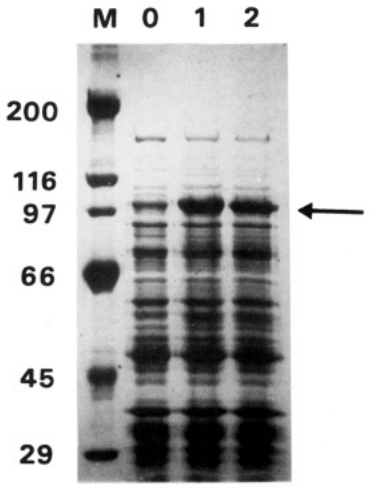

FIGURE 5: Expression of truncated MLCK mutants in *E. coli*. *E. coli* strain BL21 (DE3) was transformed with mutated plasmids to produce MLCK mutants MT799 and MT796. Each transformant was grown in LB medium with ampicillin, and the expression of the mutants was induced by IPTG: lane M, molecular mass marker; lane 0, cell lysate just before induction; lanes 1 and 2, cell lysate 6 h after induction. Mutants MT799 and MT796 are shown by an arrow in lanes 1 and 2, respectively.

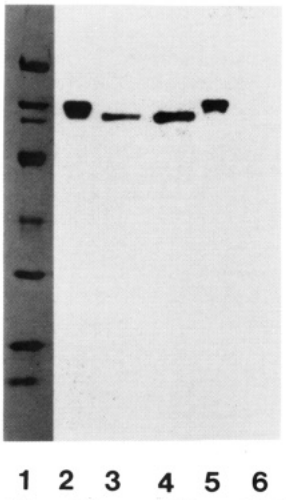

FIGURE 6: Western blot of the recombinant MLCK expressed in *E. coli*. After 6 h of IPTG induction, total cell homogenates were applied to SDS–PAGE, and then the proteins were electroblotted onto Immobilon P sheets as described in Materials and Methods: lane 1, molecular mass standards stained with Coomassie Brilliant Blue; lane 2, naturally isolated MLCK; lane 3, MT799; lane 4, MT796; lane 5, wild-type recombinant MLCK; lane 6, BL21 cell extract without containing MLCK expression vector.

Western blotting analysis of the active fraction (Figure 7, inset). Neither MT799 nor MT796 bound to the calmodulin affinity column. This is consistent with the three-dimensional structural analysis results that tryptophan 800 and leucine 813 are the anchoring sites for calmodulin binding (Ikura et al., 1992). The MLCK activities of the purified MT799 and MT796 were both very low in the presence or absence of Ca^{2+}/calmodulin (Table I); however, as shown in Figure 8, the MLCK activity of both the MT799 and MT796 mutants was activated markedly by tryptic proteolysis. The MLCK activity induced by proteolysis was observed in the absence of Ca^{2+}/calmodulin (Figure 8), and the addition of Ca^{2+}/calmodulin did not cause further activation. K_{LC} values of the Ca^{2+}/calmodulin-independent kinase produced were 0.10 and 0.16 mg/mL for MT799 and MT796, respectively, which were similar to those for natural MLCK (K_{LC}= 0.16 mg/mL) and recombinant wild-type MLCK (K_{LC} = 0.16 mg/mL).

The time course of the activation of MT799 and MT796 by tryptic proteolysis was similar to that for the production of Ca^{2+}/calmodulin-independent kinase activity of naturally isolated gizzard MLCK (Figure 8). It was previously shown (Ikebe et al., 1987) that proteolysis of MLCK in the absence of Ca^{2+}/calmodulin causes a rapid decrease in calmodulin-dependent activity, followed by a gradual increase in MLCK activity which becomes Ca^{2+}/calmodulin-independent. This is due to the production of the 64-kDa inactive fragment and the 61-kDa Ca^{2+}/calmodulin-independent fragment of MLCK, respectively. Figure 8 also shows the peptide pattern of the tryptic digests of MLCK at various times of digestion monitored by Western blotting analysis. Tryptic proteolysis of the naturally isolated MLCK initially produced a 64-kDa fragment, which converted to a 61-kDa fragment (Figure 8) as previously reported (Ikebe et al., 1987). The Ca^{2+}/calmodulin-independent kinase activity developed while the 61-kDa fragment was produced. On the other hand, the 100-kDa MLCK peptide of both the MT799 and MT796 mutants was initially digested by trypsin to produce a 64-kDa fragment, and this was further proteolyzed to produce a 61-kDa fragment with prolonged digestion. The production of the Ca^{2+}/calmodulin-independent kinase activity of the mutants correlated with the appearance of the 61-kDa fragment. The results clearly show that both MT799 and MT796 are inhibited forms of MLCK, i.e., the entire inhibitory region is present in the mutants. This implies that a cluster of the basic residues, Arg797–Arg798–Lys799, which has been proposed for the essential portion of a pseudosubstrate inhibitor sequence, is not necessary to convert MLCK into the inhibited form.

DISCUSSION

Full-length cDNA of chicken gizzard smooth muscle MLCK was expressed efficiently in the *E. coli* expression system, and the recombinant wild-type MLCK obtained had a Ca^{2+}/calmodulin-dependent activity comparable to that of naturally isolated MLCK, suggesting that the recombinant MLCK was folded properly in the *E. coli* cell so as to carry the native conformation. Therefore, we employed this expression system to produce the mutant MLCKs and thus investigate the boundary of the autoinhibitory region of smooth muscle MLCK. The mutants produced were MT799 and MT796, which contain amino acid residues 1–799 and 1–796, respectively. Kemp and co-workers (1987) have found that the MLCK sequence Ser787–Lys802 is homologous to that of the N-terminal region of the 20-kDa myosin light chain (see Table II). The sequence is characterized by an abundance of basic residues, and by aligning His805 of MLCK with Ser19 (primary phosphorylation site by MLCK) of the 20-kDa myosin light chain, it was found that Lys802, Lys799, Arg798, Arg797, Lys792, and Arg790 of smooth muscle MLCK line up with the light-chain residues Arg16, Arg13, Lys12, Lys11, Lys6, and Arg4, respectively. According to this finding, Kemp and co-workers (1987) proposed that the region 787–802 functions as a pseudosubstrate inhibitor for MLCK activity. Among the residues in this region was a cluster of basic residues Arg797–Lys799 that was thought to be essential for the pseudosubstrate inhibitor sequence (Kemp et al., 1987; Ho et al., 1991; Pearson et al., 1991).

The objective of the present study was to examine whether or not a cluster of basic residues, Arg797–Arg798–Lys799 (thought to be the fundamental requirement for the pseudosubstrate inhibitor), is necessary for the inhibition of MLCK activity. The results obtained in this study showed that both MT799 containing Arg797–Arg798–Lys799 and MT796 not

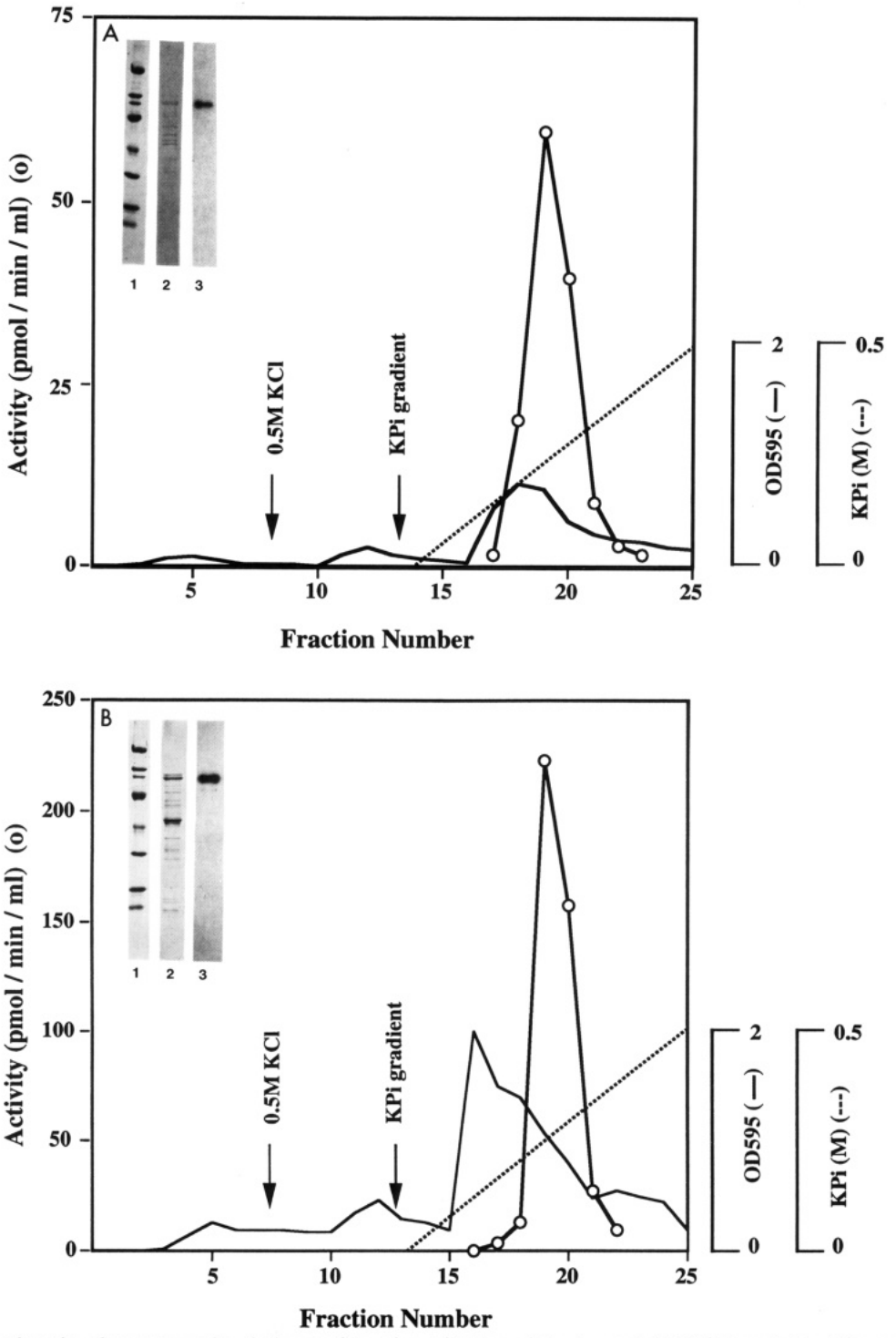

FIGURE 7: Hydroxylapatite chromatography elution profiles of purification of the truncated MLCK mutants. The mutant MLCK was extracted and partially purified by DEAE–Sephacel chromatography as described in Materials and Methods. The active fraction was then loaded on a hydroxylappatite column (1.0 × 15 cm). The flow rate was 20 mL/h, and the fractions (4.0 mL/tube) were collected. Protein elution was monitored by the Bradford method (Bradford, 1976). The kinase activity was measured as described in Materials and Methods. (A) Elution profile of MT799. The inset shows SDS–PAGE (lanes 1 and 2) and Western blots (lane 3): lane 1, molecular mass standards; lanes 2 and 3, fraction 19. (B) Elution profile of MT796. The inset shows SDS–PAGE (lanes 1 and 2) and Western blot (lane 3): lane 1, molecular mass standards; lanes 2 and 3, fraction 19.

containing Arg797–Arg798–Lys799 expressed practically no MLCK activity, that is, the kinase is inhibited. The tryptic proteolysis, which removed additional residues in the autoinhibitory region, resulted in the production of Ca^{2+}/calmodulin-independent MLCK activity (Figures 6 and 7). The results clearly showed that a cluster of the basic residues, Arg797–Arg798–Lys799, is not necessary to keep MLCK in the inhibited form. It was previously suggested (Ikebe et al., 1989) that the C-terminus of the 64-kDa inactive fragment of MLCK produced by tryptic proteolysis of the native MLCK is either Arg797 or Lys793. The present results are consistent with this earlier finding.

Although the possibility that the truncation mutants may be misfolded cannot be completely excluded and, therefore, the activation obtained by proteolysis of the mutants may be different from the activation obtained by proteolysis of the natural MLCK, this is unlikely due to the following reason. It is known that the tryptic proteolysis of naturally isolated

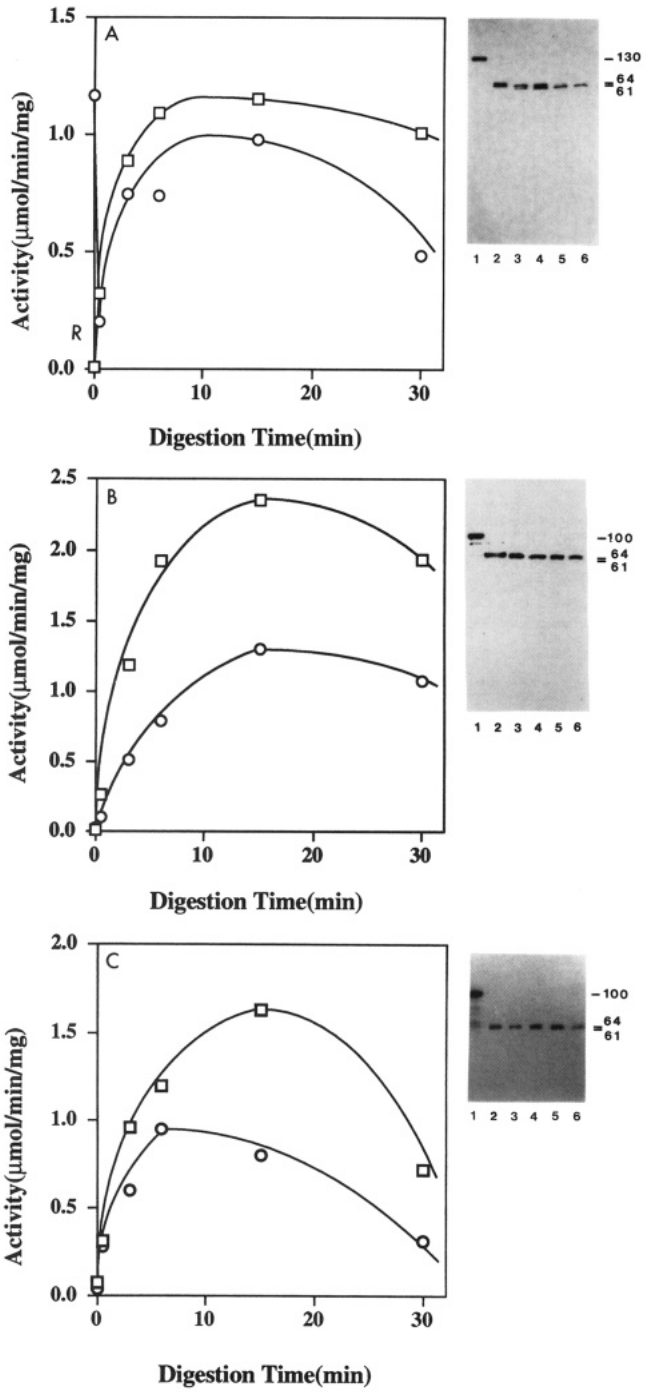

FIGURE 8: Time course of trypsin proteolysis of naturally isolated MLCK and the truncated mutants. (A) Trypsin proteolysis of naturally isolated MLCK. Kinase activity was determined as described in Materials and Methods. MLCK was digested with 20 μg/mL trypsin in the buffer containing 1 mM EGTA, 30 mM NaCl, and 30 mM Tris-HCl (pH 7.5) at 25 °C for various times. The digestion was stopped by adding 30 μg/mL soybean trypsin inhibitor. An aliquot was taken and subjected to the kinase activity assay as described in Materials and Methods. ○, 0.2 mM $CaCl_2$ and 0.25 μM calmodulin; □, 1 mM EGTA. The right panel shows immunoblotting of the proteolyzed MLCK. MLCK was digested by trypsin as described above, and then the digest was subjected to SDS–PAGE. The protein in the gel was transblotted and detected by immunostaining using LKH7 anti-MLCK monoclonal antibody. Digestion times; 0, 0.5, 3, 6, 15, and 30 min for lanes 1–6, respectively. (B) Trypsin proteolysis of MT799: ○, 0.2 mM $CaCl_2$ and 0.25 μM calmodulin; □, 1 mM EGTA. The right panel shows immunoblotting of the proteolyzed MT799. Digestion times: 0, 0.5, 3, 6, 15, and 30 min for lanes 1–6, respectively. (C) Trypsin proteolysis of MT796: ○, 0.2 mM $CaCl_2$ and 0.25 μM calmodulin; □, 1 mM EGTA. The right panel shows immunoblotting of the proteolyzed MT796. Digestion times: 0, 0.5, 3, 6, 15, and 30 min for lanes 1–6, respectively.

Table II: Sequence of Comparison of the Autoinhibitory Region of MLCK and the N-Terminal Sequence of LC_{20}

	amino acid sequence[a]
MLCK (783–807)	A **K K** L S **K** D **R** M **K K** Y M A **R R K** W Q **K** T G H A V (* *)
LC_{20} (1–21)	S S **K R A K A K** T T **K K R** P Q **R** A T S N V

[a] The *'s denote the phosphorylation sites. Basic residues are shown in boldface type.

MLCK first produces the 64-kDa inactive kinase due to the rapid proteolysis at the N-terminal region of MLCK and the regulatory region. Trypsin then hydrolyzes slowly at the C-terminal side of Lys783 to produce constitutively active enzyme. The proteolysis profile of the truncated MLCK (MT799 and MT796) was similar to that of native MLCK, i.e., MT799 and MT796 were initially proteolyzed to produce the 64-kDa fragment due to the fast proteolysis at the N-terminal region and then this was slowly converted to the 61-kDa fragment. The kinase activity was accompanied by the production of the 61-kDa fragment. Misfolding of the protein usually results in significant differences in the limited proteolysis profile; however, the proteolysis profile of the truncated kinase was quite similar to that of native MLCK. If the C-terminal region of the truncated MLCK is misfolding, it is more likely that the truncated MLCK shows constitutively active kinase activity rather than inhibited activity, since an improper conformation of the autoinhibitory region likely results in failure to interact with the catalytic domain, which leads to the production of constitutively active enzyme.

Ito et al. (1991) previously showed that a truncated mutant of MLCK, Leu447–Trp800, expressed in *E. coli* showed significant kinase activity (100 nmol/min·mg) in the absence of Ca^{2+}/calmodulin and was further activated (~3 times) by tryptic proteolysis. In contrast, MT799 and MT796 in the present study were markedly inhibited, similar to the 64-kDa inactive fragment produced from the natural MLCK by tryptic digestion (Ikebe et al., 1987). Furthermore, the MLCK activity of the mutants after tryptic proteolysis was much higher than that reported by Ito et al. (1991) and comparable to that of the naturally isolated MLCK (Table I). The difference in the properties of the recombinant MLCK can be explained by the following: (1) deletion of residues 1–446 alters the conformation of MLCK and attenuates the inhibitory effects of the autoinhibitory region, and (2) because protein synthesis started from residue 447, protein folding may be altered from the native folding.

The boundary of the N-terminal side of the autoinhibitory region of smooth muscle MLCK has been studied by using limited proteolysis of the native enzyme and by introducing stop codons in the cDNA of MLCK. Ikebe et al. (1989) reported that the C-terminal of the 61-kDa constitutively active smooth muscle MLCK fragment is Lys776. Shoemaker et al. (1990) expressed non-muscle MLCK and found that the truncated mutant (1–1067) exhibits constitutively active kinase activity. Residue 1067 of non-muscle MLCK corresponds to Glu782 of smooth muscle MLCK. Therefore, the present data, in combination with the previous findings, suggest that the autoinhibitory region of smooth muscle MLCK is defined as residues 783–796.

The residues important for the regulatory activity of MLCK have also been studied by site-directed mutagenesis. The substitution of the basic residues of skeletal muscle MLCK (Lys572–Lys573 to Gln residues, Lys577–Arg578–Arg579 to Glu–Glu–Asp, and Lys581–Lys582 to Gln residues) did not produce the constitutively active kinase, although the latter

two mutations reduced the affinity for calmodulin (Herring, 1991). Recently, Fitzsimons et al. (1992) produced a variety of MLCK mutants of rabbit uterine smooth muscle in which the basic residues in the regulatory domain from Lys961 to Lys979 (corresponding to Lys784–Lys802 in gizzard MLCK) were substituted by acidic residues. None of the mutants showed constitutively active enzyme activity, although some of these mutants altered the calmodulin-binding affinity. Among them was a mutant in which all of the basic residues between Lys961 and Lys970 (corresponding to Lys784–Lys793 in gizzard MLCK) were substituted for acidic residues, and the mutant was found to be catalytically inactive. On the other hand, Shoemaker et al. (1990) reported that the same multiple charge reversal substitutions at the corresponding region of non-muscle MLCK (rMLCK17) resulted in the constitutively active MLCK activity. The results obtained by Shoemaker et al. (1990) are consistent with the present results in which the autoinhibitory region resides at the N-terminal side of Arg797–Arg798–Lys799. The apparent discrepancy in the charge reversal substitution requires further study; however, it is possible that rMLCK17 activity is significantly lower than that of the native kinase since the absolute activity of rMLCK17 was not reported. Ito et al. (1991) reported that the truncation of MLCK at Lys793 produced constitutively active enzyme, while the truncation at Trp800 resulted in the production of a partially inhibited form (activity was inhibited 3-fold). However, kinases produced by Ito et al. (1991) do not contain the N-terminal 46-kDa portion of the molecule and could have been structurally altered as discussed above. Actually, the kinase activity of MT799, which contains the N-terminal portion of the molecule, was completely inhibited in contrast to the activity of the mutant 447–800 produced by Ito et al. (1991). Therefore, it is obscure whether or not the truncation at Lys793 produces the fully constitutively active kinase and requires further study.

The present data suggest that a cluster of basic amino acid residues, Arg797–Arg798–Lys799, which are thought to be essential for a pseudosubstrate inhibitor, is not required for maintaining MLCK in the inhibited form. This implies that the regulation of MLCK may not be explained by the pseudosubstrate inhibition mechanism originally proposed (Kemp et al., 1987). However, this sequence serves as an intramolecular inhibitor.

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