Pseudosubstrate sequence may not be critical for autoinhibition of smooth muscle myosin light chain kinase

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It has been hypothesized that basic residues in the autoinhibitory region of myosin light chain (MLC) kinase, which resemble the substrate sequence, interact with the catalytic core via charge interaction and thus inhibit the kinase activity (pseudosubstrate inhibitory hypothesis). In the present study, we produced seven MLC kinase mutants in which the residues in the autoinhibitory region are deleted to various extents, and determined the residues crucial for the autoinhibition of the kinase activity. The activities of MT799 (1–799) and MT796 (1–796) were completely inhibited, whereas MT793 (1–793), MT791 (1–791), MT787 (1–787) and MT783 (1–783) were constitutively active. The tryptic proteolysis of MT799 and MT796 activated the kinase activity, presumably due to the removal of the residues essential for autoinhibition. The mutants which showed the constitutively active kinase activity were not further activated by tryptic proteolysis, suggesting that the residues crucial for autoinhibition were already deleted. On the other hand, MT795 (1–795) was partially constitutively active (33% of maximum activity) and the tryptic proteolysis further activated the enzyme activity, suggesting that MT795 loses part of the residues essential for autoinhibition. The substitution of the residues Tyr794–Met795 but not Lys793 of untruncated MLC kinase significantly increased the Ca²⁺/calmodulin-independent kinase activity. These results clearly show that the region Tyr794–Met795–Ala796 is critical for autoinhibition. This study shows that the pseudosubstrate sequence is not critical for the autoinhibition mechanism of MLC kinase.

Key words: calmodulin/myosin light chain kinase/phosphorylation/smooth muscle

Introduction

Myosin light chain (MLC) kinase is the Ca²⁺/calmodulin-dependent protein kinase which specifically phosphorylates the regulatory light chain of myosin. In smooth muscle, the phosphorylation of the regulatory light chain of myosin is a prerequisite for the activation of actomyosin ATPase activity and thus for contraction (Hartshorne, 1987; Sellers and Adelstein, 1987). The complete amino acid sequence of MLC kinase has been determined from

skeletal muscle (Takio *et al.*, 1986; Roush *et al.*, 1988; Herring *et al.*, 1990), smooth muscle (Olson *et al.*, 1990; Gallagher *et al.*, 1991) and fibroblast (Shoemaker *et al.*, 1990). The primary structure of skeletal MLC kinase is quite distinct from the other two and is produced from a different gene, although there is significant homology among them at the catalytic core and the regulatory domain. The smooth muscle and non-muscle MLC kinase cDNA share the same nucleotide sequence except their 5' portion. Non-muscle MLC kinase cDNA contains the entire open reading frame of smooth muscle MLC kinase with an additional unique segment at the 5' side, suggesting that these two MLC kinases are derived from the same gene with different transcription initiation sites. Smooth muscle MLC kinase contains the catalytic core at the central portion of the molecule, as determined by the sequence homology conserved among various protein kinases (Olson *et al.*, 1990; Gallagher *et al.*, 1991) as well as by direct functional analysis (Ikebe *et al.*, 1987). The calmodulin binding region was first demonstrated by isolating the calmodulin binding peptide (Lukas *et al.*, 1986) and subsequently by analyzing the enzyme activity of the truncation or amino acid residue substitution mutants of MLC kinase (Bagchi *et al.*, 1989). It is located at the C-terminal side of the catalytic domain. The mechanism of calmodulin binding to MLC kinase was recently revealed by analysis of the three-dimensional structure of the calmodulin–MLC kinase peptide complex with multidimensional NMR spectroscopy (Ikura *et al.*, 1992; Meador *et al.*, 1992). It was found that two hydrophobic residues 13 residues apart (Trp800 and Leu813) are the essential calmodulin binding sites which anchor the MLC kinase to the C-terminal and N-terminal hydrophobic parts of calmodulin. The interaction involves a large number of Met residues of calmodulin (Ikura *et al.*, 1992). The interaction between calmodulin and MLC kinase is also supplemented by a number of possible electrostatic interactions.

Of interest is how the binding of calmodulin activates MLC kinase. It was shown that the proteolysis of smooth muscle MLC kinase first yields a 66 kDa calmodulin-dependent active fragment which can be converted to a 64 kDa inactive fragment then a 61 kDa constitutively active fragment by further proteolysis (Ikebe *et al.*, 1987, 1989). This indicated the existence of the autoinhibitory region toward the N-terminus of the calmodulin binding site. Supporting this notion, it was also shown that the synthetic peptide modeled after the N-terminal side of the calmodulin binding region of MLC kinase strongly inhibited both native MLC kinase activity (Kemp *et al.*, 1987) and constitutively active MLC kinase (Ikebe *et al.*, 1987).

An interesting feature of the structure of the autoinhibitory region is that the sequence contains an extremely

high number of basic residues. Kemp *et al.* (1987) found that there is homology between the amino acid sequence of the autoinhibitory region of MLC kinase and that of myosin regulatory light chain N-terminal to the phosphorylation site (Ser19 of the light chain) in terms of the alignment of the basic residues, and proposed that the inhibition of MLC kinase is achieved by a pseudosubstrate inhibitor mechanism. Among the basic residues of MLC kinase matching with the basic residues of the light chain, a cluster of basic residues of MLC kinase (Arg797–Lys799) was suggested to be important (Kemp *et al.*, 1987; Pearson *et al.*, 1988, 1991). Using synthetic peptide analogs, a cluster of basic residues of MLC (Lys11–Arg16) was also found to be important for a substrate recognition site since the deletion or substitution of these residues significantly decreases the substrate activity (Kemp *et al.*, 1983; Kemp and Pearson, 1985).

Recently, the three-dimensional structure of the catalytic core of smooth muscle MLC kinase was modeled from the crystal structure of the complex of the cAMP-dependent protein kinase catalytic subunit and the inhibitor peptide (Knighton *et al.*, 1992). The possible interaction between the basic residues in the autoinhibitory domain and the acidic residues in the catalytic domain was suggested. In the present study, we produced various truncated and substituted mutant MLC kinases by means of recombinant DNA technology, and studied the regulatory properties of the mutant MLC kinases in order to understand the structural nature of the autoinhibition of MLC kinase. The results showed that the sequence Tyr794–Ala796, but not the basic residues, is critical for the autoinhibition of MLC kinase, therefore MLC kinase autoinhibition is not achieved by a pseudosubstrate inhibitor mechanism.

Results

A series of mutant MLC kinase constructs (Figure 1), in which the amino acid residues in the autoinhibitory domain are eliminated to variable extents, were made by means of an *in vitro* mutagenesis strategy (see Materials and methods). It has been suggested that the residues Ala783–Gly804 (Figure 1) comprise the autoinhibitory domain of smooth muscle MLC kinase. Within this span, 10 basic amino acid residues are present, and it has been hypothesized that these residues play an essential role in the autoinhibition of the kinase (see Discussion). Therefore, we focused our attention on this region and produced six truncated mutant MLC kinases which contain various lengths of the autoinhibitory region (Figure 1). Figure 2A shows the SDS–PAGE profiles of the cell lysates. For all truncated MLC kinase mutant constructs, a single polypeptide of apparent M_r of 100 kDa, which was not found in untransformed BL21 cell lysate, appeared after 1- β -D-thiogalactopyranoside (IPTG) induction. The expression of the 100 kDa peptide was not found before IPTG induction, suggesting that the 100 kDa peptide was the recombinant MLC kinase (data not shown). We performed Western blotting analysis to confirm the authenticity of the peptides. We used LKH7 as an antibody probe since this antibody recognizes the catalytic domain of the kinase (Araki and Ikebe, 1991); therefore, it is expected to recognize any proteolytic fragments which lose either the N- or C-terminal domain of the kinase.

Fig. 1. Functional domain of smooth muscle MLC kinase and the structure of various mutants of MLC kinase. Top: a schematic representation of the domain organization of smooth muscle MLC kinase. The inhibitory domain and the calmodulin binding domain are defined according to Kemp *et al.* (1987) and Lukas *et al.* (1986), respectively. (*) indicates the two calmodulin anchoring hydrophobic residues (Ikura *et al.*, 1992; Meador *et al.*, 1992). Bottom: the amino acid sequence of the truncated mutants of MLC kinase tested in this study. The nomenclature of the mutated kinases is given on the left.

Actually, LKH7 recognized various proteolyzed fragments of MLC kinase as shown in Figure 2C. It was shown that the 100 kDa peptide was specifically recognized by an anti-MLC kinase monoclonal antibody (Figure 2B). This analysis also indicated that the expressed MLC kinase mutants were not degraded into smaller peptides but were stably expressed in the cells. On the other hand, a peptide with an apparent M_r of 130 kDa was expressed for the wild-type MLC kinase construct. Again, no degradation of the kinase was observed, as judged from Western blotting analysis (Figure 2).

The recombinant MLC kinase was extracted from the cells by sonication. The majority of the expressed MLC kinase mutants were recovered in the supernatant (data not shown). The extracted MLC kinase was purified by DEAE–Sephacel column chromatography. During this purification step, not only the bacteria oriented proteins but also the bulk of the non-protein cellular material was separated from the recombinant MLC kinase. The MLC kinase mutants were eluted from the column at ~0.2 M NaCl and these partially purified MLC kinase fractions were used for the experiments. Figure 3 shows the Western blotting analysis of the partially purified MLC kinase mutants. For all mutant MLC kinases, there was virtually no proteolytic degradation observed during the purification steps.

Both MT799 and MT796 showed very low MLC kinase

Fig. 2. SDS-PAGE and Western blot of the truncated MLC kinase mutants expressed in *E. coli*. After 6 h of IPTG induction, total cell homogenates were applied to SDS-PAGE. The gel was stained with Coomassie brilliant blue (A). The gel was also subjected to electroblotting to transfer the proteins to Immobilon P sheets (Millipore) and the proteins were detected by immunostaining using LKH7 anti-MLC kinase monoclonal antibody which recognizes the catalytic domain of the kinase (B) (Araki and Ikebe, 1991) (see Materials and methods). Western blot of the proteolytic fragments of MLC kinase (C). MLC kinase (0.5 mg/ml) was digested with trypsin (10 µg/ml) at 25°C for various times then the reaction was stopped with 100 µg/ml of soybean trypsin inhibitor. The sample was subjected to SDS-PAGE followed by Western blotting analysis using LKH7 anti-MLC kinase antibody. Digestion times are 0, 0.5, 1, 2, 5 and 15 min for lanes 1, 2, 3, 4, 5 and 6, respectively. A and B: lane 1, BL21 cell without MLC kinase expression vector; lane 2, wild-type recombinant MLC kinase; lane 3, MT799; lane 4, MT796; lane 5, MT795; lane 6, MT793; lane 7, MT791; lane 8, MT783. Molecular mass is indicated on the left. Arrows indicate the wild-type MLC kinase and mutant MLC kinases, respectively.

Fig. 3. Western blot of the purified MLC kinase mutants. The MLC kinase mutants were purified as described in Materials and methods. The Western blotting was done as described in Figure 2. Lane 1, MT796; lane 2, MT795; lane 3, MT793; lane 4, MT791; lane 5, MT783. Molecular mass is indicated on the left. The arrow indicates the mutant MLC kinases.

Table I. Specific activities of recombinant MLC kinases^a

	Undigested		Tryptic digested ^b	
	EGTA	Ca ²⁺ /CaM	EGTA	Ca ²⁺ /CaM
Natural MLCK	0.02	1.17	1.15	0.98
r-wild MLCK ^c	0.02	1.10	1.02	0.90
MT799	0.01	0.01	1.25	1.13
MT796	0.05	0.04	0.96	0.97
MT795	0.35	0.30	1.05	0.95
MT793	0.55	0.53	—	—
MT791	0.65	0.60	—	—
MT783	0.60	0.54	—	—

^aThe activities (µmol/min/mg) were determined in the presence of 30 mM Tris-HCl (pH 7.5), 30 mM NaCl, 4 mM MgCl₂, 0.1 mM [γ -³²P]ATP, 0.1 mg/ml LC20 and 1 mM EGTA or 0.2 mM CaCl₂/5 µg/ml calmodulin (CaM) at 25°C.

^bThe activities shown here are maximum values after tryptic proteolysis.

^cr-wild; recombinant wild.

activity (Table I, Figure 4), but the activity was markedly increased by tryptic proteolysis (Figure 4). These results suggest that MT799 and MT796 are the inhibited forms of MLC kinase and that proteolysis converted these mutants from the inhibited form to the constitutively active form by eliminating the amino acid residues at the N-terminal side of the autoinhibitory region. In contrast, MT793, MT791, MT787 and MT783 were constitutively active and the tryptic proteolysis did not increase the kinase activity but rather decreased it (Figure 4). These results clearly showed that these truncated mutant MLC kinases were the constitutively active form and, therefore, the amino acid residues critical for the autoinhibition of MLC kinase were deleted. The decrease in the activity by tryptic proteolysis with prolonged digestion was also found for the proteolysis of MT796 (Figure 4) and for the natural MLC kinase (Ikebe *et al.*, 1989) which is due to the further proteolysis of the catalytic domain to form smaller pieces. It is noted that the activity of the constitutively active mutants was somewhat lower than the maximum activity obtained by tryptic proteolysis of the inactive mutants, i.e. MT799 and MT796. This may be due to the stability of the constitutively active MLC kinase since it

Fig. 4. Effect of trypsin proteolysis on the activity of MLC kinase mutants. MLC kinase was digested with 20 μ g/ml trypsin in the buffer containing 30 mM Tris-HCl (pH 7.5), 1 mM EGTA and 30 mM NaCl at 25°C for various times. The digestion was stopped by adding 100 μ g/ml soybean trypsin inhibitor. An aliquot was taken and subjected to the MLC kinase activity assay as described in Materials and methods. The activity is expressed as % of the maximum activity which is 0.96, 1.05, 0.55 and 0.65 μ mol/min/mg for MT796, MT795, MT793 and MT791, respectively. (A) MT796; (B) MT795; (C) MT793; (D) MT791.

is known that the 61 kDa constitutively active MLC kinase fragment produced from naturally isolated MLC kinase gradually decreases its activity with time (Ikebe *et al.*, 1987).

On the other hand, MT795 was half way between the two groups of MLC kinase mutants. The activity of MT795 was partially inhibited and the tryptic proteolysis further activated 2–3 times, suggesting that the elimination of Ala796 partially abolished the autoinhibitory activity of MLC kinase. The difference in the properties of these mutant MLC kinases is due to the difference in the designed constructs (difference in the expected amino acid sequence) but not due to possible proteolysis during the preparation or expression, because no visible proteolytic products of MLC kinase were observed in both total cell homogenate (Figure 2) and the purified MLC kinase preparations (Figure 3). Based upon the results obtained, it is concluded that the sequence Tyr794–Ala796 is critical for the autoinhibitory activity of MLC kinase.

None of the mutant MLC kinases tested showed Ca^{2+} /calmodulin dependence for their activity (Table I). Consistent with this, all truncated MLC kinases did not bind to a calmodulin affinity column. This is reasonable because

recent three-dimensional structural analysis revealed that Trp800 and Leu813 of smooth muscle MLC kinase are essential for the binding of MLC kinase to calmodulin (Ikura *et al.*, 1992; Meador *et al.*, 1992) and these residues are not included in the mutants produced here. To further evaluate the importance of the region Tyr794–Ala796, we produced mutants having all the MLC kinase residues but with substituted residues in the above region. *Escherichia coli* BL21 was transformed with a MLC kinase mutant expression vector and the mutant proteins were expressed by IPTG induction. As shown in Figure 5, a polypeptide with an apparent M_r of 130 kDa, which corresponds to MLC kinase, was observed for all lysates of the transformed cells.

The 130 kDa band was recognized by anti-MLC kinase monoclonal antibody, showing that this band represents the recombinant MLC kinase mutants. Western blotting analysis also revealed that there was no degradation of MLC kinase (Figure 5). Since all three mutants contain the calmodulin binding region, MLC kinase mutants were purified by calmodulin–Sepharose 4B affinity chromatography and used for kinase activity. No degradation products were produced during the purification steps, as

Fig. 5. SDS-PAGE and Western blot of the MLC kinase substitution mutants expressed in *E. coli*. MLC kinase was expressed as described in Figure 2. The expression of the recombinant MLC kinase analyzed by Coomassie blue staining of the gel as well as by Western blotting as described in Figure 2. (A) SDS-PAGE of total cell homogenate. Lane 1, naturally isolated gizzard MLC kinase; lane C, BL21 cell without MLC kinase expression vector; lane 2, Y794E; lane 3, M795E; lane 4, M795G; lane 5, M795K; lane 6, A796G; lane 7, YM794/795DG; lane 8, YMA794-796DGD; lane 9, YM794/795GG; lane 10, YMA794-796GGD; lane 11, YM794/795GR; lane 12, K793E; lane 13, K793V. (B) Western blot of total cell homogenate. Lanes 1-13 are as described above.

judged from Western blotting analysis of the purified MLC kinases. The purified MLC kinase mutants were subjected to the kinase activity assay. While the activity in the presence of Ca^{2+} /calmodulin was not significantly altered, the Ca^{2+} /calmodulin-independent kinase activity was significantly changed by the mutation (Figure 6). The single substitution of Tyr794 or Met795 increased the Ca^{2+} /calmodulin-independent activity 5–10 times. The increase in the Ca^{2+} /calmodulin-independent kinase activity was more pronounced when both Tyr794 and Met795 were substituted, and the activity was increased by ~100 times. On the other hand, the substitution of Lys793 by Glu or Val did not alter the Ca^{2+} /calmodulin-dependent activity, suggesting that the change in the activity by amino acid substitution is site specific. These results, combined with the results of the truncation mutants, clearly indicate that the residues Tyr794–Ala796, but not the basic residues, are critical for the autoinhibitory activity of MLC kinase.

The K_{LC20} and K_{ATP} for MLC kinase are shown in Table II. The values were not significantly different between the various mutants and natural/wild-type MLC kinases, indicating that the affinity for the 20 000 Da light chain

Fig. 6. MLC kinase activity of MLC kinase substitution mutants in the absence of Ca^{2+} /calmodulin. The activity of the various substitution mutants in the absence of Ca^{2+} /calmodulin was compared with that of wild-type MLC kinase. The kinase activity of wild-type kinase in the absence of Ca^{2+} /calmodulin was ~0.3% of the maximum activity.

Table II. K_{LC20} and K_{ATP} for recombinant MLC kinases

	K_{LC20} (mg/ml) ^a	K_{ATP} (μM) ^a
Natural MLCK	0.13	59.8
r-wild MLCK	0.11	46.0
MT799	0.11	39.0
MT796	0.16	26.4
MT795	0.16	28.5
MT793	0.26	69.6
MT791	0.15	73.7
MT783	0.13	33.3

^a K_{LC20} and K_{ATP} were determined as described in Materials and methods.

and ATP of the kinase was unaffected by the deletion of the calmodulin binding domain. To see whether or not the charge interaction governs the autoinhibition mechanism, the MLC kinase activity of the inhibited form was examined as a function of ionic strength (Figure 7). The activity did not increase but rather decreased with increasing NaCl concentration. The results suggest that the charge interaction is not the major determinant for the autoinhibition mechanism.

Discussion

It has been noted that MLC kinase contains the autoinhibitory region towards the N-terminus of the calmodulin binding region (Ikebe *et al.*, 1987, 1989; Kemp *et al.*, 1987; Pearson *et al.*, 1988). The autoinhibitory region is characterized by an unusually high content of basic amino acid residues and, because the basic residues in the autoinhibitory region are aligned well with the basic residues present at the N-terminal side of the 20 000 Da MLC, it has been hypothesized (Kemp *et al.*, 1987;

Fig. 7. NaCl dependence of MLC kinase activity. The MLC kinase activity was measured as described in Materials and methods. ▼, native MLC kinase in the presence of 5 μ g/ml calmodulin and 0.2 mM CaCl_2 ; ▲, native MLC kinase in the presence of 1 mM EGTA. ■ and ●, MT796 and MT795 in the presence of 1 mM EGTA, respectively.

Pearson *et al.*, 1988) that these basic amino acid residues play a crucial role in the autoinhibition of the kinase via a pseudosubstrate inhibitor mechanism. The present results clearly show that the residues Tyr794–Ala796, but not the basic residues, play an important role in the autoinhibition of MLC kinase.

Ito *et al.* (1991) previously showed that a truncated smooth muscle MLC kinase, Leu447–Trp800, expressed in *E. coli* is the inhibited form, because further proteolysis somewhat activated (~3-fold) the kinase activity. This construct exhibited significant Ca^{2+} /calmodulin-independent activity (100 nmol/min/mg), presumably due to the deletion of the N-terminal 446 residues which may alter the conformation of MLC kinase or folding of MLC kinase during protein synthesis. Quite recently, Yano *et al.* (1993) showed that the activity of the deletion mutant MLC kinase (1–796) is completely inhibited and the further deletion of the N-terminal side of the autoinhibitory domain by tryptic proteolysis is required for the constitutively active kinase. The boundary of the N-terminal side of the autoinhibitory region of smooth muscle MLC kinase has been studied. Ikebe *et al.* (1989) reported that the C-terminus of the 61 kDa constitutively active kinase fragment is Lys776. Shoemaker *et al.* (1990) reported that the truncated mutant (1–1067) of non-muscle MLC kinase shows constitutively active kinase activity (residue 1067 of non-muscle MLC kinase corresponds to Glu782 of smooth muscle MLC kinase). The present results are consistent with these earlier results and further define the residues critical for the autoinhibitory activity as Tyr794–Met795–Ala796. Interestingly, the basic residues which are supposed to be important according to the pseudosubstrate inhibitor hypothesis were not involved in the autoinhibition of the kinase, but the hydrophobic residues are present in this region.

The notion that charge interaction may not dominate the interaction between the autoinhibitory region and the catalytic domain is also supported by the result that an increase in the ionic strength did not attenuate the autoinhibition of the kinase. The present results are also consistent with earlier studies in which the basic residues

were substituted by non-charged or acidic residues. The substitution of the basic residues of skeletal MLC kinase within the autoinhibitory domain (Lys572–Lys573 to Gln residues, Lys577–Arg798–Arg 579 to Glu–Glu–Asp and Lys581–Lys582 to Gln residues) failed to produce constitutively active kinase (Herring, 1991). For smooth muscle MLC kinase, the basic residues in the regulatory domain of rabbit uterine MLC kinase were substituted by acidic residues, but none of the substitutions produced the constitutively active kinase (Fitzsimons *et al.*, 1992). Consistent with these reports, the substitution of Lys793 by either Glu or Val showed no change in the Ca^{2+} /calmodulin-independent activity (Figure 6). In contrast, the substitution of Tyr794 and Met795 significantly increased the Ca^{2+} /calmodulin-independent activity. Therefore, our results, along with the previous reports, lead to the conclusion that the basic residues in the autoinhibitory region do not play a predominant role in the autoinhibition of MLC kinase.

Quite recently, Gallagher *et al.* (1993) reported that the substitution of the various acidic residues in the catalytic core by non-charged or basic residues results in the increase in affinity for calmodulin, although none of the mutants showed the constitutively active kinase activity. Based upon this result, it was proposed that the charge interaction at the various sites contributes to the interaction between the catalytic core and the regulatory domain. We cannot exclude the possibility that the charge interaction may also contribute to some extent to the stabilization of the interaction between the autoinhibitory site and the catalytic domain, although such an interaction would be rather supplementary.

In the native enzyme, the residues critical for autoinhibition are assumed to interact with the catalytic domain of MLC kinase, thus inhibiting the catalytic activity. The direct interaction between the catalytic site and the regulatory site is supported by the recent finding that MLC kinase intramolecularly autophosphorylates at its regulatory domain, i.e. Thr803 (Tokui *et al.*, 1995), suggesting that the catalytic site is in close proximity to the regulatory site. Therefore, it is possible that the binding of calmodulin abolishes the interaction between the catalytic site and the autoinhibitory site, thus activating the MLC kinase activity. Although the mechanism by which calmodulin binding cancels the autoinhibitory activity is still unknown and requires further study, it is of interest that one of the two calmodulin anchoring hydrophobic sites (Trp800) (Ikura *et al.*, 1992; Meador *et al.*, 1992) is just 4–6 residues downstream of the critical residues of the autoinhibitory sites identified in this study. It is possible that the hydrophobic region of calmodulin might compete with the catalytic domain for interaction with the hydrophobic region of the autoinhibitory region, i.e. Tyr794–Ala796.

Materials and methods

Protein preparation and biochemical procedures

Naturally isolated smooth muscle MLC kinase was prepared from frozen turkey gizzard (Ikebe *et al.*, 1987) and calmodulin was prepared from bull testes (Walsh *et al.*, 1983). The recombinant 20 000 Da light chain of myosin was prepared as described previously (Kamisoyama *et al.*, 1994) and used as a substrate. MLC kinase activity was measured at 25°C using the recombinant light chain (0.1 mg/ml) as a substrate in

Table III. Oligonucleotide primers for site-directed mutagenesis

MLC Kinase Mutants	Oligonucleotide Primers ^a
	K N M E A K K L S K D R M K K Y M A R R K W Q K T G H 5' CCAAAAACATGGAAGCCAAAAAATTTCCTCAAGATAGGATGAAGAAATATATGGCCAGAAGAAAATGGCAGAAAACAGGCCATG
MT799	GCCAGAAGAAAA TGATAG AAAAACAGGCCATG
MT796	GAAGAAATATATGGCC TGATGA AAATGGCAGAAAAAC
MT795	GGATGAAGAAATATATG TAG AGAAGAAAATGGCAG
MT793	GATAGGATGAAGAAA TAGTAG GCCAGAAGAAAATG
MT791	CCAAAGATAGGATG TAGTAA TATATGGCCAGAAG
MT783	CCAAAAACATGGAAGCC TAATAA CTTTCCTCAAGATAGG

^aMutated nucleotides are indicated by bold letters. Stop codons are boxed.

30 mM NaCl, 4 mM MgCl₂, 0.2 mM CaCl₂, 5 µg/ml calmodulin 0.1 mM [γ -³²P]ATP and 30 mM Tris-HCl (pH 7.5). Ca²⁺/calmodulin-independent kinase activity was estimated in 1 mM EGTA without addition of Ca²⁺/calmodulin. The amount of ³²P incorporated into the light chain was measured as described by Walsh *et al.* (1983).

SDS-PAGE was carried out on 7.5–20% polyacrylamide gradient gels by using the discontinuous buffer system of Laemmli (1970). The following M_r standards were used: 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).

Western blotting was carried out as described previously (Higashihara *et al.*, 1989; Yano *et al.*, 1993), using the monoclonal antibody termed LKH7 which recognizes the catalytic core of MLC kinase as a primary antibody (Araki and Ikebe, 1991). The bound antibodies were detected by the enhanced chemiluminescence method (Amersham).

The concentration of MLC kinase in partially purified recombinant MLC kinase mutants was estimated from densitometric analysis (Hoefer Scientific Instruments GS 300) of the Western blot stained by the enhanced chemiluminescence detection method. Purified natural MLC kinase of a known concentration was subjected to a series of dilutions and the diluted MLC kinase was analyzed by Western blotting. MLC kinase was then quantitated by densitometric analysis, as described above, and used as a standard for estimation of the recombinant MLC kinase concentration.

Molecular biological procedures

The MLC kinase cDNA (3 kb) which contains the open reading frame, except 150 bp at the 5' end, was obtained from a chicken gizzard cDNA library as described previously (Yano *et al.*, 1993). The missing 150 bp of MLC kinase cDNA were synthesized by reverse transcription from chicken gizzard total RNA and amplified by PCR as described (Yano *et al.*, 1993). These cDNA fragments were in-frame ligated and inserted into the pT7-7 expression vector to produce wild-type MLC kinase expression vector pTML3 (Yano *et al.*, 1993).

To produce the various truncated and substituted MLC kinase mutants (Figure 1), stop codons were introduced at appropriate positions or particular amino acid residues were changed by a site-directed mutagenesis strategy (Table III). For the mutagenesis, a transformer Site-Directed Mutagenesis Kit (Clontech Inc.) was used. Complementary oligonucleotides containing termination codons or changed amino acid residue codons were made and used as mutagenesis primers (Table III). Another oligonucleotide primer (Δ BamHI), 5'-CGCGCCCCGGGG-ATAATCTAGAGTCGACC-3', with the mutated nucleotides underlined, was also made to eliminate the unique BamHI in the MLC kinase expression vector at the multicloning (selection primer) site. A pair of oligonucleotide primers, a mutagenesis primer and a selection primer, were annealed to the template, pTML3, and the *in vitro* mutagenesis was performed as described previously (Yano *et al.*, 1993). The mutation of MLC kinase cDNA was confirmed by sequence analysis (Sanger *et al.*, 1977).

Expression and purification of recombinant MLC kinase

Escherichia coli strain BL21 (DE3) was transformed with pTML3 or the plasmid containing the mutant cDNAs of MLC kinase. The transformants were grown in LB medium with 50 µg/ml ampicillin until the OD₆₀₀ reached 1.2. IPTG (final concentration of 0.5 mM) was added to the culture and harvested 6 h after induction. Cells were collected by centrifugation at 4000 g for 10 min and stored in -80°C. The expressed MLC kinase was stable during the storage. For preparation, the packed cells were washed with 30 mM Tris-HCl (pH 7.5) then sonicated in volumes of lysis buffer [15 mM Tris-HCl (pH 8.3), 2 mM DTT, 5 mM EDTA, 10 µg/ml leupeptin and 1 mM PMSF] for 5 min on ice. After sonication, the sample was centrifuged in 1.5 ml microtubes at 10 000 g for 15 min, then the supernatant was subjected to the subsequent purification steps.

The purification of wild-type MLC kinase was done as described previously (Yano *et al.*, 1993). For the mutant MLC kinases, the extract was applied to a DEAE-Sephacel column (1.5 cm×20 cm) equilibrated with 30 mM Tris-HCl (pH 8.3), 1 mM EGTA, 1 mM DTT and 0.1 mM PMSF. The mutant MLC kinase was eluted by a linear NaCl gradient (0–0.5 M). The flow rate was 30 ml/h, and 5 ml/tube fractions were collected. The mutant MLC kinase was eluted at ~0.2 M NaCl. The elution of MLC kinase was monitored by both Western blotting analysis and the enzyme activity assay. For MT795, MT793, MT791, MT787 and MT783, the kinase activity was measured in the solution containing 1 mM EGTA, 4 mM MgCl₂, 50 mM NaCl, 30 mM Tris-HCl (pH 7.5), 0.1 mM [γ -³²P]ATP and 0.1 mg/ml 20 000 Da MLC at 25°C. For MT799 and MT796, the fractions were first digested with 20 µg/ml trypsin (TPCK treated, Worthington) in buffer containing 1 mM EGTA, 30 mM NaCl and 30 mM Tris-HCl (pH 7.5) at 25°C for 10 min, and the reaction was stopped by adding 100 µg/ml soybean trypsin inhibitor (Sigma). The activity of the fractions was then measured as described above.

Acknowledgements

This work is supported by American Heart Association Grant in Aid and by NIH grant HL 47530 and HL 37117.

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Received on December 15, 1994; revised on March 13, 1995