Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 3187–3191, May 1985
Biochemistry

Identification of the calmodulin-binding domain of skeletal muscle myosin light chain kinase

(protein phosphorylation/Ca^{2+}-dependent enzyme regulation/synthetic peptide)

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ABSTRACT **In the course of determining the primary structure of rabbit skeletal muscle myosin light chain kinase (MLCK; ATP:protein phosphotransferase, EC 2.7.1.37) a peptide fragment was obtained that appears to represent the calmodulin-binding domain of this enzyme. Low concentrations of the peptide inhibited calmodulin activation of MLCK ($K_i \simeq 1$ nM). The peptide was not associated with a catalytically active, calmodulin-independent form of MLCK that was obtained by limited proteolysis. The peptide is 27 residues in length and represents the carboxyl terminus of MLCK. The sequence of the peptide shows no significant homology with any known protein sequence. The peptide contains one tryptophanyl residue and a high percentage of basic and hydrophobic residues, but no acidic or prolyl residues. Much of the sequence has a high probability of forming α helix. A chemically synthesized peptide has been prepared to study the interactions of the peptide and calmodulin in more detail. The intrinsic tryptophan fluorescence of the synthetic peptide shows a significant enhancement ($\approx$45%) in the presence of Ca^{2+} and calmodulin; fluorescence enhancement is maximal at a peptide:calmodulin stoichiometry of 1:1. Calmodulin-Sepharose affinity chromatography in the presence of 2 M urea indicates that the interaction of peptide and calmodulin is Ca^{2+}-dependent. The results of these studies indicate that the catalytic and calmodulin-binding domains of MLCK represent distinct and separable regions of the protein. In addition, the results provide a basis for future studies of the molecular and evolutionary details of calmodulin-dependent enzyme regulation.**

Calmodulin is a Ca^{2+}-binding protein that is ubiquitously distributed and highly conserved throughout eukaryotic evolution. Although it is known to regulate many Ca^{2+}-dependent enzymes (recently reviewed in ref. 1), little is known about the interactions of calmodulin with these proteins at the molecular level. Myosin light chain kinase (MLCK; ATP:protein phosphotransferase, EC 2.7.1.37) is one of the better characterized calmodulin-regulated enzymes (reviewed in refs. 2 and 3) and is found in both muscle and nonmuscle tissues. The enzyme occurs in tissue-specific forms, which vary widely in molecular weight, antigenic determinants, and enzymatic properties (3, 4). It catalyzes the phosphorylation of a specific class of myosin light chain subunit, termed the P-light chain (5). In smooth muscle this phosphorylation is required for initiation of contraction (reviewed in refs. 2 and 6), whereas in skeletal muscle the phosphorylation appears to modulate the degree of tension produced during isometric contraction (3, 7).

A determination of the amino acid sequence of rabbit skeletal muscle MLCK was undertaken by these several laboratories as part of a long-range study of protein kinase and calmodulin-dependent enzyme structure. One of the goals in this endeavor was to identify the calmodulin-binding domain of this enzyme. The sequence and preliminary characterization of a peptide derived from MLCK that displays the properties expected of a calmodulin-binding domain are described in this report. This information should prove useful in understanding the evolutionary relationships of calmodulin-dependent enzymes as well as the molecular mechanisms of calmodulin action.

METHODS

MLCK was purified from fresh rabbit skeletal muscle by a procedure similar to that described by Nagamoto and Yagi (8), details of which will be published elsewhere. The enzyme was subjected to limited proteolysis in order to generate lower molecular weight forms that retained catalytic activity (unpublished data). With trypsin, a mixture of two forms with M_rs of approximately 60,000 and 40,000 by NaDodSO$_4$/PAGE was generated. The mixture, which contained nearly equal amounts of the two forms, was completely calmodulin-dependent and was termed T60/40. Evidence was obtained that the M_r 60,000 form was an intermediate form that could be further degraded to the M_r 40,000 form. A calmodulin-independent form of MLCK was generated by limited proteolysis with chymotrypsin. This form had a M_r of 35,000 by NaDodSO$_4$/PAGE and showed identical catalytic activity in the presence of either 2 mM EGTA or 0.2 mM Ca^{2+} and 5 μM calmodulin. This calmodulin-independent form of MLCK was denoted C35. Details of the preparation and properties of these proteolyzed forms of MLCK will be described elsewhere. CNBr digestions of intact MLCK, T60/40, and C35 were performed following reduction and *S*-carboxymethylation as described by Takio *et al.* (9). After lyophilization, the CNBr digests were either dissolved in water to assay for MLCK inhibition or fractionated by a combination of size exclusion and reversed-phase high-pressure liquid chromatography (HPLC). Details of peptide isolation and characterization will be presented elsewhere.

Calmodulin was prepared from bovine testis by using batchwise DEAE-cellulose (DE-52, Whatman) chromatography, phenyl-Sepharose (Sigma) chromatography (10), and gel filtration chromatography (Bio-Gel A-0.5m, Bio-Rad). Mixed myosin light chains from rabbit skeletal muscle were prepared as described (11). Standard solid-phase methods (12) were used for synthesizing the calmodulin-binding peptide. The composition and sequence of the peptide were verified by amino acid analysis, Edman degradations, and proton NMR spectroscopy. [γ-^{32}P]ATP was obtained from New England Nuclear.

Calmodulin-binding activity of peptides and CNBr digests

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Abbreviations: MLCK, myosin light chain kinase; Mops, 4-morpholinepropanesulfonic acid.

was determined by assaying for their ability to inhibit calmodulin-dependent MLCK activity. MLCK activity was determined as described (11). All reaction mixtures (50 μl final volume) contained 200 μM $CaCl_2$, 10 mM magnesium acetate, 2 mM [γ-^{32}P]ATP (≈200 cpm/pmol), 15 mM 2-mercaptoethanol, 50 mM 4-morpholinepropanesulfonic acid (Mops) at pH 7.0. Concentrations of proteins and peptides are indicated in the figure legends. The following catalytic rates were determined for use in calculating apparent K_i values: v_0, the catalytic rate in the absence of any inhibitor or exogenously added calmodulin (sufficient endogenous calmodulin was present in the myosin light chain preparation to give values for v_0/V_{max} of 0.3–0.4); v_i, the catalytic rate in the presence of inhibitor and no exogenously added calmodulin; V_{max}, the catalytic rate in the presence of 1 μM exogenously added calmodulin. The apparent K_i value for a given inhibitor was calculated by using the equation:

$$K_i = \frac{[I]}{\frac{(V_{max}/v_i) - 1}{(V_{max}/v_0) - 1} - 1} - \frac{K_{CaM}}{V_{max}/v_i - 1},$$

where [I] is the inhibitor concentration and K_{CaM} is the concentration of calmodulin required for half-maximal activation (a value of 1.0 nM was used for K_{CaM} in all calculations). The equation assumes simple competition for Ca_4^{2+}·calmodulin between the inhibitor and MLCK. The K_i values stated in the text were calculated only for $0.1 < v_i/v_0 < 0.9$.

RESULTS

In the course of the primary structure determination of rabbit skeletal muscle MLCK, it was of interest to know whether any fragments of the enzyme retained the ability to bind calmodulin. Digestion of the enzyme with CNBr yielded a number of moderate-size fragments that might be expected to retain some affinity for calmodulin. To assay various digests and isolated fragments for calmodulin-binding activity, the ability of a sample to inhibit MLCK activity was measured in a reaction mixture in which calmodulin was limiting (see *Methods*). As shown in Fig. 1, an unfractionated CNBr digest of MLCK was inhibitory at low concentrations. The apparent K_i value was calculated to be 4.5 nM. This value is in good agreement with the activation (0.3 nM) and binding (3 nM) constants reported for MLCK interaction with calmodulin (13, 14). To establish whether loss of calmodulin sensitivity was related to the loss of a calmodulin-binding fragment, CNBr digests of various proteolyzed forms of MLCK were assayed for inhibitory activity (Fig. 1; see *Methods* for description of nomenclature). The unfractionated CNBr digest of T60/40 (a form which retains calmodulin dependence) was inhibitory at low concentrations (K_i = 1.3 nM). The CNBr digest of C35 (a form lacking calmodulin dependence), however, showed a marked reduction (by a factor of 38) in the ability to inhibit (K_i = 170 nM). These data are consistent with there being a high-affinity calmodulin-binding fragment in CNBr digests of calmodulin-dependent forms of MLCK (intact MLCK and T60/40), which is of much lower affinity and/or quantity in CNBr digests of a calmodulin-independent form of the enzyme (C35).

Assays of several isolated CNBr fragments (Fig. 2) indicated that one peptide, termed M13, exhibited nearly all of the inhibitory capacity (K_i = 9 nM) of the unfractionated digest. Two other fragments, M11–12 and M12, showed no inhibitory ability. Peptide maps of CNBr digests of MLCK and C35 indicated that M13 was degraded to a much shorter form in C35, whereas fragment M12 was found in digests of both C35 and MLCK (unpublished observations). A peptide corresponding to the sequence of M13 (see Fig. 3 for sequence) but lacking a carboxyl terminus homoserine was synthesized and assayed for its ability to inhibit calmodulin-dependent MLCK activity. This synthetic peptide showed the highest potency (K_i = 0.9 nM) of any material tested (data not shown). The lower inhibitory potency of M13 as isolated from the CNBr digest was probably due to side reactions of sensitive side chains (e.g., tryptophan oxidation, asparagine deamidation), which tend to occur under the relatively harsh conditions of CNBr digestion and the subsequent purification procedures. When high concentrations of calmodulin (1 μM) were included in the assay, the same MLCK activity was observed in the presence or absence of synthetic M13 (40 nM), indicating that the mechanism of peptide inhibition was through calmodulin and not by a direct effect on MLCK (data not shown).

The sequence of the inhibitory peptide contains a tryptophanyl residue; therefore, it was of interest to investigate the interaction of the synthetic inhibitory peptide with calmodulin by using the intrinsic fluorescence of the peptide (Fig. 4). Fluorescence enhancement was maximal (≈45%) at a molar ratio of 1 mol of peptide per mol of calmodulin, consistent with the stoichiometry of interaction between intact MLCK and calmodulin (8, 11, 14, 19–22). Similar increases in tryp-

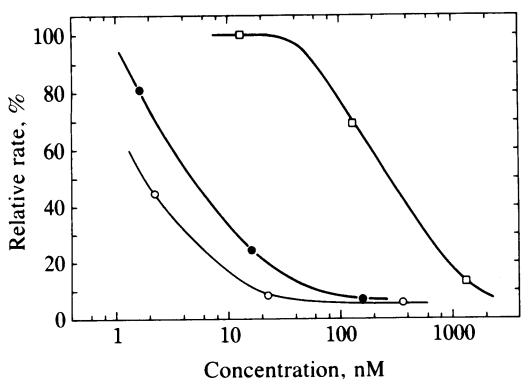

FIG. 1. Inhibition of calmodulin-dependent MLCK activity by various unfractionated CNBr digests of MLCK. CNBr digests of intact MLCK (●), T60/40 (○), and C35 (□) were prepared and assayed for their ability to inhibit calmodulin-sensitive MLCK activity. Indicated concentrations were determined by amino acid analysis assuming M_rs of 81,000, 40,000, and 35,000 for intact MLCK, T60/40, and C35, respectively. The concentrations of enzyme and myosin light chains were 0.78 nM and 47 μM, respectively. Catalytic rates are expressed relative to v_0.

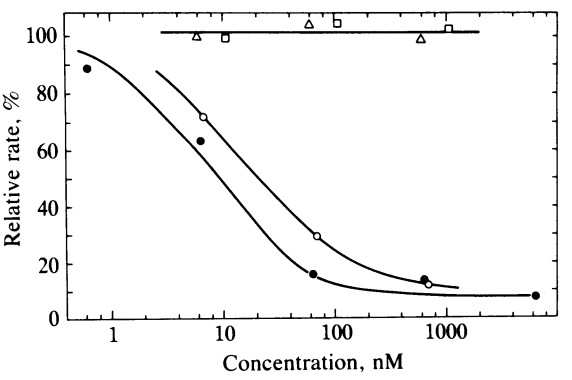

FIG. 2. Inhibition of calmodulin-dependent MLCK activity by isolated CNBr fragments of MLCK. CNBr fragments M11–12 (△), M12 (□), and M13 (○) were quantitated by amino acid analysis. An unfractionated CNBr digest of MLCK (●) was prepared and quantitated as described in the legend to Fig. 1. The concentrations of enzyme and myosin light chains were 1.6 nM and 94 μM, respectively. Catalytic rates are expressed relative to v_0.

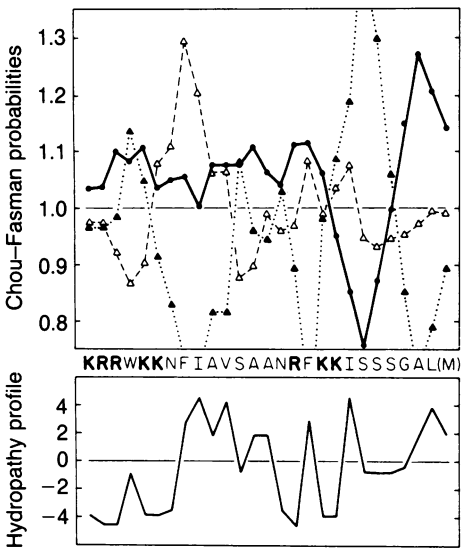

FIG. 3. Structural profile of the calmodulin-binding peptide derived from MLCK. (*Upper*) Chou–Fasman (15) probabilities for α helix (●—●), β strand (△---△), and β turn (▲···▲), averaged over four residues using the program of Corrigan and Huang (16) and data from Argos *et al.* (17). (*Lower*) Hydropathy profile of the sequence using the values reported in Kyte and Doolittle (18). The hydropathy values were not averaged. The peptide sequence is shown between the panels using the one-letter code described in the legend to Table 1. The basic residues, lysine (K) and arginine (R), are indicated in boldface to emphasize their positions in the sequence. The carboxyl-terminal methionine (M) is not present in the synthetic peptide.

tophan fluorescence intensity were reported for MLCK interaction with calmodulin (21). Because of the relatively high concentrations of peptide and calmodulin required for fluorometric measurements (1.3 μM), accurate determinations of binding affinities cannot be obtained; however, the fluorescence data indicate that the interaction of peptide and calmodulin is of high affinity (K_d < 10 nM), consistent with the affinity determined by the inhibition assay.

To determine whether the interaction of the peptide with calmodulin is Ca^{2+}-dependent, chromatography of the peptide on calmodulin-Sepharose was employed. Calmodulin was coupled to CNBr-activated Sepharose 4B (Pharmacia) to the extent of ≈5 mg of calmodulin per ml of packed Sepharose (23). The column (0.6 × 8 cm) was equilibrated in 2 M urea/0.2 M ammonium acetate, pH 6.8/0.2 mM $CaCl_2$, and 40 nmol of synthetic peptide was applied (flow rate = 250 μl/min). Ten column volumes (20 ml) of flow-through fraction was collected. Ten column volumes of elution buffer (2 M urea/0.2 M ammonium acetate, pH 6.8/2 mM EDTA) was then applied to the column and the column effluent was collected. To quantitate the peptide in each fraction, aliquots were analyzed by reversed-phase HPLC (Vydac C-4 analytical column) using a linear gradient from 0.1% trifluoroacetic acid to 0.1% trifluoroacetic acid/50% acetonitrile. The peptide was detected by UV absorbance (206 nm) and tryptophan fluorescence (excitation, 280 nm; emission, >300 nm), identified by its elution time, and quantitated by absorbance peak height. Only 1% of the applied peptide was present in the calmodulin-Sepharose flow-through fraction, but 86% of the peptide was recovered upon elution with EDTA. Thus, the peptide interacts with calmodulin in a Ca^{2+}-dependent manner even in the presence of 2 M urea. When these experiments were performed in the absence of urea, only a small percentage of applied peptide (3%) eluted with 2 mM EDTA, indicating that a denaturant is required for quantitative dissociation from calmodulin-Sepharose. Since denaturing conditions are not required for elution of intact MLCK from calmodulin-Sepharose, these experiments indicate differences in the way that the peptide and the whole protein interact with calmodulin.

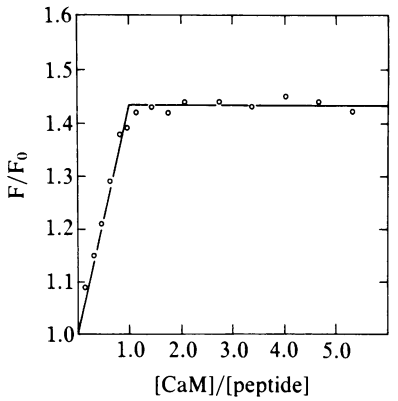

FIG. 4. Stoichiometry of calmodulin–peptide interaction as determined by peptide intrinsic tryptophan fluorescence. Synthetic peptide (1.3 μM) corresponding to M13 was titrated with calmodulin (CaM) in 0.2 mM $CaCl_2$/20 mM Mops, pH 7.0/100 mM NaCl. Tryptophan emission was monitored at 355 nm with an excitation wavelength of 295 nm by using a Perkin-Elmer MPF-44A recording spectrofluorometer. Concentrations of peptide and calmodulin were determined by UV absorption with the molar extinction coefficients ε_{280} = 5555 and ε_{276} = 3300, respectively. The intersecting lines were drawn to best fit the points between $0 \leq$ [CaM]/[peptide] ≤ 1.0 and $1.0 \leq$ [CaM]/[peptide] ≤ 5.3, respectively. Data are expressed as the ratio of observed fluorescence (F) to peptide fluorescence in the absence of calmodulin (F_0).

DISCUSSION

Although calmodulin is known to regulate a large number of enzyme activities, little is known about the interactions between calmodulin and its target enzymes at the molecular level. One reason is the lack of information regarding the amino acid sequences of calmodulin-dependent enzymes. One of our primary objectives in undertaking the sequence determination of MLCK was to identify the sequence of its calmodulin-binding domain. We report here evidence to indicate that we have isolated a calmodulin-binding fragment from MLCK that displays the properties expected of a bona fide calmodulin-binding domain.

A number of criteria should be satisfied by a peptide fragment that is thought to act as the calmodulin-binding domain of a calmodulin-dependent enzyme. The peptide M13 isolated from MLCK fulfills the following criteria: (*i*) it is present in forms of the enzyme known to be calmodulin-dependent but absent from a form that is calmodulin-independent; (*ii*) it has a binding affinity for calmodulin comparable to that of the intact enzyme; (*iii*) its stoichiometry of interaction with calmodulin is identical to that of the intact enzyme; (*iv*) its interaction with calmodulin is Ca^{2+}-dependent; (*v*) its interaction with calmodulin prevents calmodulin's interaction with the enzyme. This list of criteria is by no means complete but rather represents a starting point for the further investigation of putative calmodulin-binding domains.

The sequence of the calmodulin-binding fragment of MLCK (Fig. 3) and its position within the overall sequence of the enzyme have been determined (unpublished observations). The peptide is 27 amino acids in length and represents the carboxyl terminus of MLCK. The sequence shows no significant homology with any protein in the current protein sequence data base (>2900 entries), as indicated by the SEARCH program (24). There is no obvious homology between M13 and either the γ subunit of phosphorylase kinase (25) or the inhibitory subunit of troponin (troponin-I; ref. 26), the only proteins in the data base known to bind calmodulin.

This lack of homology may reflect either convergent evolution of calmodulin-binding domains in these different proteins or different modes of interaction between each protein and calmodulin. Although troponin-I has been used as a model calmodulin-binding protein because of its relatively high affinity for calmodulin (3 nM; ref. 27), it is thought to be associated with troponin-C rather than calmodulin *in vivo*. The interaction of calmodulin with the γ subunit of phosphorylase kinase is also different than with MLCK in that calmodulin is an integral subunit of native phosphorylase kinase and is not readily dissociated from the enzyme in the absence of Ca^{2+} (28). The calmodulin-dependent enzymes that most resemble MLCK in their mode of interaction with calmodulin (e.g., cyclic nucleotide phosphodiesterase, calcineurin, adenylate cyclase, plasma membrane Ca^{2+}-pump ATPase, etc.) may be more interesting to compare with M13 as sequence data become available.

The primary structure of the calmodulin-binding peptide (Fig. 3) has several interesting features that may relate to its calmodulin-binding function. It contains a high percentage of basic residues (30%) but has no acidic or prolyl residues. The basic residues are grouped in clusters with five near the amino terminus of the peptide and three near the middle. These residues are very likely to be involved in the interaction of MLCK with calmodulin, since calmodulin is quite acidic and ionic interactions are known to be important in this interaction (13). Between the two clusters of basic residues is a highly hydrophobic region (Fig. 3), which is also probably important in the interaction of MLCK and calmodulin since hydrophobic interactions are known to be involved in the interactions of calmodulin with MLCK and other target enzymes (13, 29, 30). The sequence also contains one tryptophanyl residue, located in the cluster of basic residues at the amino terminus. The fluorescence of this amino acid is increased upon interaction with calmodulin (Fig. 4), suggesting that this particular tryptophan residue may have been involved in the fluorescence changes observed by Johnson *et al.* (21) in their studies of MLCK–calmodulin interactions. Secondary structural predictions based on the technique of Chou and Fasman (15, 16) indicate that the calmodulin-binding peptide of MLCK has a high probability of forming α helix over much of its length (Fig. 3).

A number of peptides have been used as models for calmodulin–target enzyme interactions because of their high affinity for calmodulin (31–33). When the sequences of these peptides are compared to that of M13, there do not appear to be any striking similarities of sequence (Table 1). However, the model peptides and M13 all share three structural features thought to be important for interactions with calmodulin: clusters of basic residues; hydrophobic residues adjacent to the basic residues; predicted or observed α-helical structure (31–35). It will be important to compare these several peptides with respect to their three-dimensional structure and with respect to their sites of interaction on calmodulin.

It is worth noting that the peptide fragment from MLCK denoted M12 (Table 1) also has many of the primary structural features expected of a calmodulin-binding peptide but shows no detectable binding activity (Fig. 2). Moreover, it is present in both calmodulin-dependent and -independent forms of MLCK (unpublished observations). The carboxyl-terminal sequence of M12 is compared to that of M13 and several model calmodulin-binding peptides in Table 1. The carboxyl terminus of M12 is basic, has a region of high hydrophobicity, and shows a high probability of forming α helix. Moreover, the sequence of M12 is quite similar to that of vasoactive intestinal peptide, which is known to interact with calmodulin with reasonably high affinity ($\approx$50 nM; ref. 36). Thus, it is interesting that this peptide (M12) does not bind to calmodulin with high affinity. Because M12 is located at the carboxyl terminus of MLCK, just to the amino terminus of M13, it may play some role in the regulation of MLCK by calmodulin; however, it does not appear to make significant contributions to the overall binding energy in the interaction of MLCK and calmodulin.

That regions of MLCK other than those represented by M13 may be important in MLCK–calmodulin interactions is suggested by the data from calmodulin-Sepharose chromatography of synthetic M13. Whereas MLCK readily and quantitatively elutes from calmodulin-Sepharose under nondenaturing conditions when free Ca^{2+} concentrations are reduced using chelator, only a small fraction of synthetic M13 elutes under these conditions (see *Results*). Both urea (2 M) and chelator are required to elute the peptide, suggesting that the sequence of M13 may lack determinants that are required for facile dissociation from calmodulin in the absence of Ca^{2+}. Comparison of peptide–calmodulin versus MLCK–calmodulin interactions will be important to determine the nature of these determinants.

The demonstration that limited proteolytic digestion of MLCK yields a catalytically active, calmodulin-insensitive fragment (C35) that is lacking a calmodulin-binding domain (Fig. 1) indicates that MLCK is a protein kinase of chimeric character (37), consisting of at least two domains (i.e., catalytic and regulatory). This idea is supported by comparison of the sequence of MLCK to other protein kinase sequences. MLCK is clearly homologous with every other member of the protein kinase family in the region of the molecule identified as the catalytic domain (unpublished observations). However, evidence is lacking for homology in the region of the calmodulin-binding domain, even in the case of the γ subunit of phosphorylase kinase, which is known to bind calmodulin (38). Since many calmodulin-dependent enzymes can be modified by proteolytic cleavage to yield calmodulin-independent forms (reviewed in ref. 1), it is likely that many calmodulin-dependent enzymes are chimeras with catalytic domains linked to calmodulin-binding domains.

Table 1. Sequence comparison of the calmodulin-binding peptide derived from MLCK (M13) with another peptide from MLCK (M12) and with several model peptides known to bind calmodulin with high affinity

Peptide	Sequence
M13	K–R–R–W–K–K–N–F–I–A–V–S–A–A–N–R–F–K–K–I–S–S–S–G–A–L–M
M12	W–L–N–N–L–A–E–K–A–K–R–C–N–R–R–L–K–S–Q–I–L–L–K–K–Y–L–M
Vasoactive intestinal peptide	H–S–D–A–V–F–T–D–N–Y–T–R–L–R–K–Q–M–A–V–K–K–Y–L–N–S–I–L–N
Mellitin	G–I–G–A–V–I–L–K–V–L–T–T–G–L–P–A–L–I–S–W–I–K–R–K–R–Q–Q–NH_2
Mastoparan	I–N–L–K–A–L–A–A–L–A–K–K–I–L–NH_2
Mastoparan X	I–N–W–K–G–I–A–A–M–A–K–K–L–L–NH_2
Polistes mastoparan	V–D–W–K–K–I–G–Q–H–I–L–S–V–L–NH_2

The peptide sequences labeled M13 and M12 are derived from rabbit skeletal muscle MLCK. The sequence shown for M12 is only that of the carboxyl-terminal 26 residues (total peptide length is 36 residues). The following one-letter codes are used to designate each amino acid: A (alanine), R (arginine), N (asparagine), D (aspartic acid), C (cysteine), Q (glutamine), E (glutamic acid), G (glycine), H (histidine), I (isoleucine), L (leucine), K (lysine), M (methionine), F (phenylalanine), P (proline), S (serine), T (threonine), W (tryptophan), Y (tyrosine), V (valine). Basic residues are underlined.

In summary, we have isolated and sequenced a peptide from MLCK that appears to represent the calmodulin-binding domain of this enzyme. A synthetic version of the peptide has been prepared to facilitate studies of the interaction of the peptide with calmodulin. The results presented here indicate that the peptide should prove useful in probing the interactions of calmodulin with its target enzymes. It is likely that other calmodulin-dependent enzymes will yield similar calmodulin-binding peptides, which will be interesting to compare from an evolutionary as well as a structural standpoint.

We wish to express our appreciation to Dr. Rachel Klevit for performing the proton NMR analysis of the synthetic peptide and for many stimulating discussions. We also acknowledge the excellent technical assistance of Mrs. Edwina Beckman, Ms. Barbara Flug, Mr. Floyd Kennedy, Ms. Maria Harrylock, and Mr. Roger Wade. Support for this work was provided by National Institutes of Health Grant GM15731. D.K.B. was supported as a Postdoctoral Fellow by the Muscular Dystrophy Association.

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