

# Use of DNA Sequence and Mutant Analyses and Antisense Oligodeoxynucleotides to Examine the Molecular Basis of Nonmuscle Myosin Light Chain Kinase Autoinhibition, Calmodulin Recognition, and Activity

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**Abstract.** The first primary structure for a nonmuscle myosin light chain kinase (nmMLCK) has been determined by elucidation of the cDNA sequence encoding the protein kinase from chicken embryo fibroblasts, and insight into the molecular mechanism of calmodulin (CaM) recognition and activation has been obtained by the use of site-specific mutagenesis and suppressor mutant analysis. Treatment of chicken and mouse fibroblasts with antisense oligodeoxynucleotides based on the cDNA sequence results in an apparent decrease in MLCK levels, an altered morphology reminiscent of that seen in *v-src*-transformed cells, and a possible effect on cell proliferation. nmMLCK is distinct from and larger than smooth muscle MLCK (smMLCK), although their extended DNA sequence identity is suggestive of a close genetic relationship not found with skeletal muscle MLCK. The analysis

of 20 mutant MLCKs indicates that the autoinhibitory and CaM recognition activities are centered in distinct but functionally coupled amino acid sequences (residues 1,068–1,080 and 1,082–1,101, respectively). Analysis of enzyme chimeras, random mutations, inverted sequences, and point mutations in the 1,082–1,101 region demonstrates its functional importance for CaM recognition but not autoinhibition. In contrast, certain mutations in the 1,068–1,080 region result in a constitutively active MLCK that still binds CaM. These results suggest that CaM/protein kinase complexes use similar structural themes to transduce calcium signals into selective biological responses, demonstrate a direct link between nmMLCK and nonmuscle cell function, and provide a firm basis for genetic studies and analyses of how nmMLCK is involved in development and cell proliferation.

**T**HE effects of changes in intracellular free calcium levels on nonmuscle cell motility, organelle movement, and cell morphology (2, 10, 20, 21, 35, 45, 53, 55, 81, 82) appear to involve, in part, the activation of the calmodulin/myosin light chain kinase (CaM/MLCK)<sup>1</sup> complex (3, 8, 10, 16, 17, 30, 35, 45, 55, 82, 84) which catalyzes the stoichiometric phosphorylation of myosin light chains at specific sites (8, 29, 77). However, our knowledge about CaM/MLCK signal transduction complexes, how they are properly assembled, and how these supramolecular structures are able to selectively transduce calcium signals into cellular responses in nonmuscle cells is incomplete. For example, the primary structure for a nmMLCK has not been described for any phylogenetic species. Relatedly, it is not known which features of nmMLCK are important in the proper recognition of CaM, such that a functional CaM/MLCK complex can be formed during the time constraints

of cellular responses. This knowledge is needed for a complete interpretation of experiments that seek to explore the possible *in vivo* roles of CaM/MLCK signal transduction complexes in nonmuscle cell responses. On a more general level, knowledge about the specific features of CaM/enzyme recognition and insight into how CaM activates an enzyme are key to understanding how intracellular calcium signals are selectively transduced into discrete cellular responses, predicting how mutations or other perturbations of CaM/enzyme complexes might alter cellular responses to environmental stimuli, and facilitating attempts at targeted drug design based on selective structural and mechanistic features.

There are at least three forms of MLCK found among animal tissues: skeletal muscle MLCK (skMLCK), smooth muscle MLCK (smMLCK) and nonmuscle MLCK (nmMLCK) (for a review see reference 72). The skMLCK has been shown (32, 33, 66, 73) to be biochemically, genetically, and physiologically distinct from smMLCK and nmMLCK. For example, the similarity between the amino acid sequence for smMLCK (23, 48, 50, 60) and that for skMLCK (33, 66, 73)

1. *Abbreviations used in this paper:* CaM, calmodulin; CEF, chick embryo fibroblast; MLCK, myosin light chain kinase; nm, nonmuscle; r, recombinant; sk, skeletal muscle; sm, smooth muscle.

is not much greater than the similarity between skMLCK and other CaM regulated protein kinases (5, 7, 11, 12, 15, 26, 46, 64). In terms of cellular physiology (for a review see reference 72), the smMLCK and nmMLCK are thought to be involved in the initiation of contraction in response to calcium signals, whereas this role is fulfilled by the troponin complex in skeletal muscle tissue. While the smMLCK and nmMLCK are similar to each other in mobility during SDS-PAGE and immunoreactivity (9, 17, 75), it is not known how they are related structurally or genetically.

Clearly, there is a need to define in more detail the molecular constituents of the nonmuscle motility systems. As an initial step towards filling this void in our knowledge, we report here the amino acid sequence of a nmMLCK (deduced from the cDNA sequence encoding the chicken embryo fibroblast protein), and the relationship of its primary structure to other CaM regulated protein kinases, especially MLCK from muscle tissues.

Our knowledge of the primary structure of a nmMLCK, and the insight provided by our demonstration that the catalytic and CaM regulatory regions of nmMLCK and smMLCK are identical in amino acid sequence, allowed us to use the information gained from previous studies (19, 23, 48, 50, 78) of smMLCK to directly test, by site-specific mutagenesis, models (4, 27, 33, 39, 42–44, 48, 50, 61) of how MLCK structure might correlate with CaM binding and enzyme activation. All of the models have in common the assumption that there are key regions within the MLCK sequence that prevent the manifestation of protein kinase activity due to an intramolecular inhibition, or autoinhibition, and that activation by CaM results from the release of this intramolecular inhibition. The models differ in whether or not they assume that the CaM binding sequences are overlapping (4, 27, 33, 42, 44, 61), or distinct (39, 50) from the autoinhibitory sequences. The most popular of these models for MLCK is the pseudosubstrate hypothesis (4, 27, 33, 42, 44, 61). A more detailed version of this hypothesis postulates that a portion of the CaM binding sequence, that has some similarity to the myosin light chain substrate, is the most critical region for maintaining autoinhibition (61). However, the results of initial deletion mutagenesis (4, 33) and recent proteolysis (39) studies of MLCK are not entirely consistent with the models for MLCK that equate the CaM binding sequences with autoinhibitory activity. Therefore, the lack of agreement between initial site-specific mutagenesis results and models of how the primary structure of MLCK is related to CaM binding and relief of autoinhibition, combined with previous differences among models, require a reappraisal of some of the fundamental assumptions upon which these models are based.

We have used the cDNA sequence of nmMLCK described in this report to test, through the use of site-specific mutagenesis, extant models about CaM recognition and relief of autoinhibition of MLCKs. The results, which include the production of a novel conformational suppressor mutation in the CaM binding sequence, are consistent with the hypothesis that the 20-residue CaM binding segment of smMLCK and nmMLCK, originally identified by chemical fragmentation and synthetic peptide analogue studies (48, 50), is quantitatively important in MLCK's selective recognition of and response to CaM. Relatedly, the data allow the tentative identification within the MLCK sequence of a CaM regula-

tory unit, which includes a new definition of the autoinhibitory region. The correlation of these structures with function and molecular mechanism has, in turn, been used as the basis for an initial examination of how nmMLCK might be involved in cell function through the use of antisense oligodeoxynucleotides on fibroblasts.

## Materials and Methods

### Protein Cleavage, Peptide Isolation, and Amino Acid Sequence Analysis

Proteolytic digestion of chicken gizzard MLCK was carried out using previously described protocols (48, 68). The peptides were isolated by HPLC (48, 68) over a Brownlee RP8 (1.0 × 250 mm) column (Brownlee Labs, Santa Clara, CA) using 0.1% trifluoroacetic acid (solvent A) and 60% acetonitrile containing 0.08% trifluoroacetic acid (solvent B).

Amino acid composition analyses were done on acid hydrolysates (6 N HCl, 20–24 h) using the Picotag (Waters Associates, Milford, MA) procedure modified as previously described (68). Automated Edman degradations were performed on a protein sequencer (model 470A; Applied Biosystems Inc., Foster City, CA) and PTH amino acids were identified by HPLC with an on-line PTH analyzer (model 120A; Applied Biosystems Inc.) according to the manufacturer's recommended and previously described (68) protocols.

### DNA Sequence Analysis

DNA sequences were obtained by using the protocols described in detail by Zimmer et al. (85). Briefly, DNA sequencing reactions were performed using a Biomek 1000 pipetting workstation (Beckman Instruments, Inc., Palo Alto, CA), commercially available fluorescent-labeled primers, and the dideoxynucleotide chain termination method (67) according to the manufacturer's (Applied Biosystems Inc.) recommended protocol. The fluorescent-labeled reaction products were analyzed on a model 370A DNA sequencer (Applied Biosystems Inc.) (13). Isotopic manual sequence using deoxyadenosine 5' [ $\alpha$ - $^{32}$ S]thiotriphosphate (>600 Ci/mmol; Amersham Corp., Arlington Heights, IL) was obtained with the Sequenase protocol (US Biochemical Corp., Cleveland, OH). Where necessary, exact-match synthetic oligodeoxynucleotide primers, made on model 380 and 381 DNA synthesizers (Applied Biosystems Inc.), were used in the manual, isotopic protocol.

### Construction and Screening of a Chicken Embryo Fibroblast cDNA Library

Total cytoplasmic RNA was isolated from secondary cultures of chicken embryo fibroblast (CEF) cells (74) using the phenol/SDS extraction protocol (70). Poly(A)<sup>+</sup>RNA was selected from the total cytoplasmic RNA by two passages over oligo-dT-cellulose (70). A  $\lambda$ gt11 cDNA library containing  $\sim 1.1 \times 10^6$  PFU was prepared commercially (Clontech Corp., San Diego, CA) by the method of Huynh et al. (38).

The approach used for screening and characterization of positive clones was the same as that previously described by Zimmer et al. (85). Briefly, two library equivalents were screened by hybridization with a  $^{32}$ P-labeled fragment (bases 2,218–2,785; Fig. 2) isolated from a partial MLCK cDNA clone (50). DNA from positive clones were characterized by restriction mapping or DNA sequence analysis. Two large inserts,  $\lambda$ 11 and  $\lambda$ 50, were obtained from this screening. These were indistinguishable by restriction mapping and DNA sequence analysis. A fragment consisting of 400 bp of the most 5' end of clone  $\lambda$ 11 (Fig. 1 A) was used to rescreen the same library, resulting in the isolation of two additional clones ( $\lambda$ 23 and  $\lambda$ 6; Fig. 1 A). These three overlapping clones were used to establish the complete DNA sequence of the region encoding fibroblast MLCK.

### Strategy for Establishing DNA Sequence of MLCK cDNA

The strategy for establishing the DNA sequence was essentially the same as that used previously for CaM genes (85) and is outlined in Fig. 1 B. Briefly, the inserts of clone  $\lambda$ 23 and  $\lambda$ 6 were subcloned directly into Bluescript II and the fragments for sequencing were obtained by nested de-

letion method of Henikoff (31) (Erase-a-Base Kit; Promega Biotech, Madison, WI). In addition, three overlapping clones were subdigested with restriction endonucleases as indicated in Fig. 1 B and subcloned into one of the following vectors: M13mp8, M13mp9, M13mpl8, or M13mpl9 (New England Biolabs, Beverly, MA); Phagescript (Stratagene, La Jolla, CA); or Bluescript II (Stratagene). These plasmids were transformed into competent *Escherichia coli* (strain UT481 or the XL1-blue strain in the case of Bluescript II). Single- and double-stranded DNA templates were prepared from the transformed bacteria essentially as described (54). Sequence data were assembled and analyzed using the PC Gene and IG-Suite software packages (Intelligenetics, Mountain View, CA).

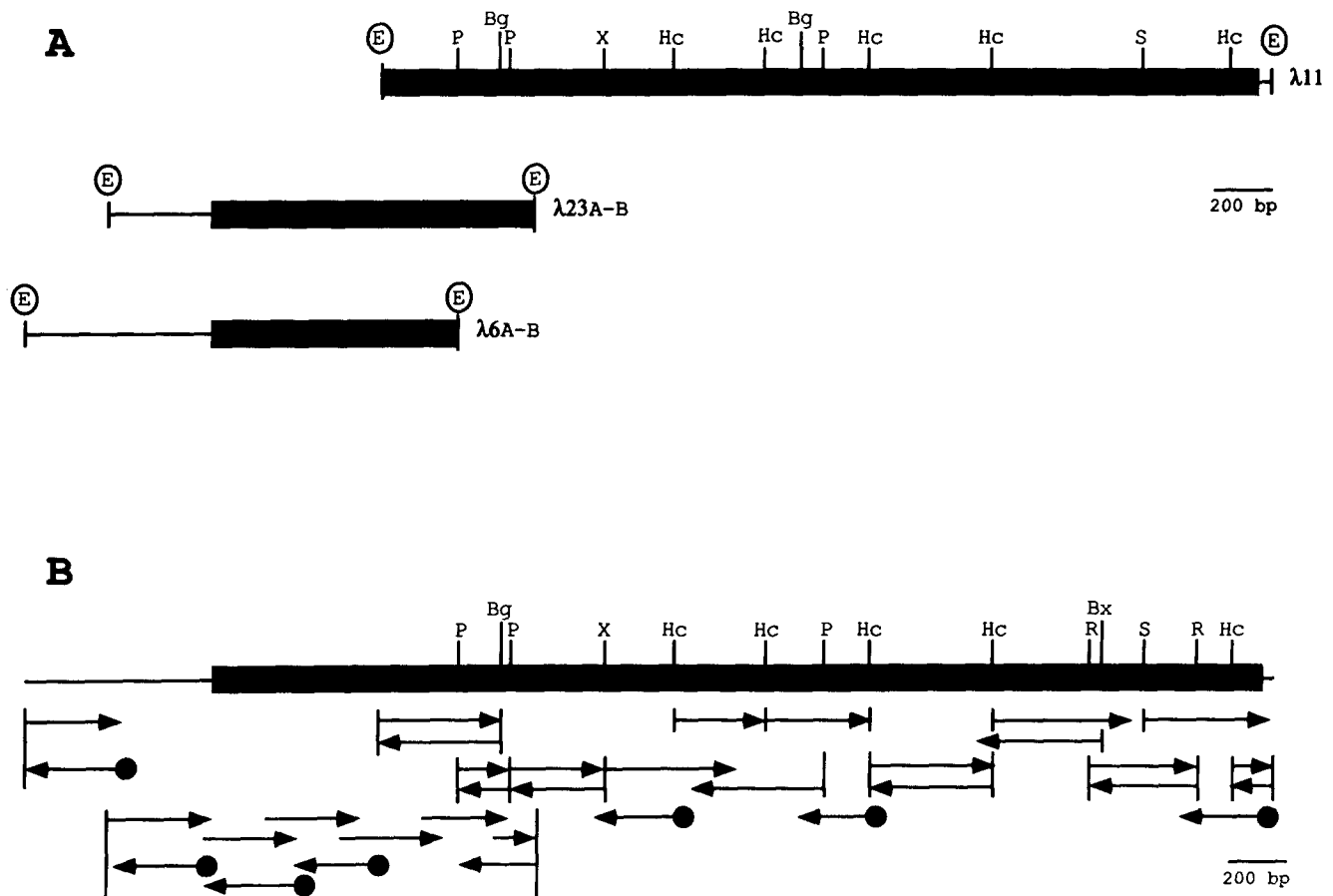
### Preparation of Recombinant DNA Encoded Proteins

The construction and verification of structure for the E84K and E120K mutant CaMs followed previously described protocols (14, 65, 80). A caltractin cDNA (36) in the expression vector pKK223-3 was obtained from Dr. Bessie Huang, Research Institute of Scripps Clinic (La Jolla, CA). Calmodulin and caltractin were purified essentially as described (37, 80). Briefly, phenyl-Sepharose (Pharmacia Inc., Piscataway, NJ) chromatography was used to adsorb proteins in buffer A (50 mM Tris-HCl, pH 7.5) con-

taining 1 mM 2-mercaptoethanol, 500 mM NaCl, 0.3 mM CaCl<sub>2</sub>. The column was washed with the same buffer, then the bound proteins eluted by changing to buffer A containing 1 mM EGTA and 0.5 mM DTT.

rMLCK1 encompasses nucleotides 2,220–4,012 (Fig. 2) of the fibroblast MLCK cDNA. The amino acid sequence of rMLCK1 minus the four amino-terminal linker amino acids is given in the top line of Fig. 3. To ligate the coding region of rMLCK1 into the expression vector pVUCH1 (49, 50) and maintain the appropriate spacing from translational elements in the expression vector, as well as maintain the reading frame, an 11-bp synthetic oligodeoxynucleotide adapter encoding four additional amino acids (Ala-Asn-Ser-Gly) at the amino terminus was used. A site-specific, cassette-based mutagenesis approach (14) was used to generate the 20 mutant constructs shown in Fig. 5. All mutations were verified by restriction mapping and DNA sequence analysis across the mutated region.

rMLCK1 and mutant derivatives of rMLCK1 were produced in *E. coli* and purified by the protocols described below. UT481 *E. coli* cells, transformed with expression plasmids, were grown in NZCYM medium until they reached an OD<sub>550</sub> of 0.5–0.7. Protein expression was induced by the addition of IPTG to a final concentration of 0.5 mM. Cells were cultivated for an additional hour, harvested, and either processed immediately or frozen at –20°C until needed. The purification protocol consisted of the following six steps.



**Figure 1.** The complete coding sequence for CEF MLCK has been determined by analysis of overlapping cDNA clones. (A) Restriction maps of DNA isolated from the bacteriophage exhibiting hybridization with fragments isolated from a partial MLCK cDNA (50) are shown. Only the segment containing the inserted cDNA molecules is diagrammed. Names of clones refer to the particular filter upon which the bacteriophage were isolated. The placement of the restriction sites was accomplished by analysis of restriction enzyme digests, as described in Materials and Methods. Overlap of individual clones was established by DNA sequence analysis. The heavy bars represent the open reading frame. The circled E indicates the location of the Eco RI sites generated as part of the linker during library construction. (B) The strategy used to establish the complete DNA sequence of the 3,774-bp open reading frame and 698-bp flanking DNA is shown. Only the minimal data necessary to establish the complete DNA sequence are shown, although additional confirmatory sequence was obtained. Sequences were obtained by using the dideoxy chain termination method (67) and universal primers, except where indicated by filled circles. Filled circles indicate sequences obtained with exact match oligodeoxynucleotide primers. Arrows with vertical bars indicate selected subcloned fragments. Plain arrows indicate clones from nested deletion experiments. Abbreviations used for restriction endonucleases are Bg, Bgl II; Bx, Bst XI; Hc, Hinc II; P, Pst I; R, Rsa I; S, Sac I; and X, Xba I.



Step 1 was cell disruption. Frozen bacterial cell pellets were thawed on ice (if necessary), resuspended in 5 vol of buffer B (50 mM Tris-HCl pH 7.5, 1 mM DTT, 1 mM EGTA, 5 mM EDTA), sonicated four times for 20 s on ice using the microtip on a sonicator (Branson Sonic Power Co., Danbury, CT). The mixture was centrifuged at 4°C (20 min, 3,000 g).

Step 2 was ammonium sulfate fractionation. The cleared lysate was adjusted to a final volume of 300 ml, brought to a final concentration of 1.4 M  $(\text{NH}_4)_2\text{SO}_4$ , stirred 20 min at 4°C, and centrifuged at 4°C (20 min, 20,000 g). The supernatant was recovered, adjusted to a final concentration of 2.1 M  $(\text{NH}_4)_2\text{SO}_4$ , stirred 20 min at 4°C, and centrifuged again (4°C, 20 min, 20,000 g). The final pellet, which contained the majority of the MLCK activity, was resuspended in buffer B.

Step 3 was phenyl-Sepharose chromatography. Clarified solutions containing enzymes were adjusted to a volume of 300 ml, brought to a final concentration of 1.4 M  $(\text{NH}_4)_2\text{SO}_4$ , stirred 20 min at 4°C, and centrifuged (4°C, 20 min, 20,000 g). The supernatant was loaded at a flow rate of 2.5 ml/min onto a phenyl-Sepharose column (10 ml bed volume) previously equilibrated with buffer C (buffer B containing 1.4 M  $(\text{NH}_4)_2\text{SO}_4$ ), and washed with 150 ml of buffer C. Proteins were eluted with a linear gradient from 1.4 to 0 M  $(\text{NH}_4)_2\text{SO}_4$  using a 650 Advanced Protein Purification Systems (Waters Associates, Inc., Rochester, MN). Fractions containing MLCK activity were pooled.

Step 4 was DEAE-Sepharose chromatography. The pooled fractions were diluted with buffer D (50 mM Tris-HCl pH 7.5, 1 mM DTT, 1 mM EGTA) to a conductivity of  $\leq 8$  mmho. The solution was loaded at a flow rate of 4 ml/min onto DEAE-Sepharose Fast Flow (Pharmacia Inc.) column (20 ml bed volume) previously equilibrated in buffer D. The column was washed with 15 vol of buffer D, and proteins eluted using a linear gradient from 0 to 700 mM NaCl. Fractions containing MLCK activity were pooled.

Step 5 was CaM-Sepharose chromatography. Fractions containing enzyme were diluted to 300 ml with buffer E (50 mM Tris-HCl pH 7.5, 1 mM DTT, 3 mM  $\text{MgCl}_2$ , 3 mM  $\text{CaCl}_2$ , 50 mM NaCl), adjusted to a final concentration of 2 mM  $\text{CaCl}_2$ , and loaded at a flow rate of 1 ml/min onto a CaM-Sepharose column (20 ml bed volume) prepared as described (52). The column was washed with 5 vol of buffer E, followed by 2.5 vol of buffer F (50 mM Tris-HCl pH 7.5, 1 mM DTT, 3 mM  $\text{MgCl}_2$ , 1 mM  $\text{CaCl}_2$ , 600 mM NaCl), and the proteins were eluted with 75 ml of buffer G (50 mM Tris-HCl, pH 7.5, 1 mM DTT, 3 mM  $\text{MgCl}_2$ , 200 mM NaCl, 2 mM EGTA). Fractions containing MLCK activity were pooled.

Step 6 was Mono-S HRS/5 (Pharmacia Inc.) cation exchange chromatography. Fractions containing MLCK activity were diluted to a final volume of 50 ml using buffer H (50 mM Hepes pH 7.0, 1 mM DTT, 1 mM EGTA), and loaded at a flow rate of 0.5 ml/min onto a Mono-S-Sepharose column. The protein was eluted using a linear gradient from 0 to 700 mM NaCl. Fractions containing MLCK activity were aliquoted and stored at  $-20^\circ\text{C}$ .

The purification of CaM binding protein kinases consisted of steps 1, 3, 5, and 6. The purification of the non-CaM binding protein kinases consisted of steps 1-4 and 6. For activity screening, purification of mutant rMLCKs consisted only of steps 1 and 5 with appropriately adjusted volumes.

### Protein Kinase Activity Assays and Gel Methods

The phosphotransferase activity of rMLCK1, chicken gizzard MLCK, and mutant protein kinases from  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  to a peptide substrate or chicken gizzard myosin light chains were determined as previously described (80). Briefly, standard assay conditions at  $25^\circ\text{C}$ , were 0.2 mM  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ , 50  $\mu\text{M}$  peptide substrate (KKRPQRATSNVFAM), 0.1 mM  $\text{CaCl}_2$ , 5 mM  $\text{MgCl}_2$ , 1 mM DTT, 0.1 mg/ml BSA, 50 mM Hepes pH 7.5, CaM as indicated, and kinase. The CaMPK-II peptide substrate (PLRRTLVA) and the cAMP-dependent protein kinase peptide substrate (LRRASLG) were used at 50  $\mu\text{M}$  where applicable. The CaMPK-II peptide substrate was a gift from Dr. Marita King (Ohio State University, Columbus, OH). The cAMP-dependent protein kinase peptide substrate was purchased from Sigma Chemical Co. (St. Louis, MO).

The apparent  $K_m$ s of the peptide substrate with rMLCK1, mutant protein kinases, and gizzard MLCK were determined at saturating levels of CaMs (wild type, E84K mutant, or E120K mutant) and 2 nM enzyme under the standard assay conditions. The concentration of the peptide substrate was varied from 6.3 to 500  $\mu\text{M}$ . The apparent  $K_m$ s were calculated with the assistance of Graph Pad (version 2 program; copyright 1985-1987 by H. J. Motulsky; distributed by Institute for Scientific Information Software, Philadelphia, PA) using intercepts of linear regression lines from double reciprocal plots according to the data treatment of Lineweaver and Burk (47).

SDS-PAGE and CaM overlays were done as described by Burgess et al. (9). Western blot analyses were done as previously described by Burgess

and co-workers (9, 75) using a polyclonal rabbit antibody against chicken gizzard MLCK.

### Computer-based Sequence Analysis

Amino acid sequence comparisons were done using the program FASTA (62). Nucleic acid or protein sequence database searches used the FASTDB program from the Intelligenetics program suite (Version 5.35; Intelligenetics, Mountainview, CA). The databases searched were Genbank release 61.0 (9/89) and PIR (Protein Identification Resource) release 21.0 (6/89). Amino acid sequence motif analysis was done with the pattern searching program QUEST (Intelligenetics). The previously described motifs of Benian et al. (6) were optimized for finding related sequences in fibroblast MLCK. Analysis of fibroblast MLCK for additional repeated motifs was initiated by manual inspection and then optimized with the QUEST program. All of the above programs were run on a SUN Microsystems computer workstation. Analysis of the fibroblast MLCK amino acid sequence for potential phosphorylation and glycosylation sites was done using the program PROSITE within the PCGENE (version 6.01) program package (Intelligenetics). Helical wheel projections and the calculation of hydrophobic moments were done with the PCGENE program HELWHEEL according to the method of Eisenberg et al. (18).

### Results

#### Chicken Fibroblast MLCK Has a Unique, Segmentally Organized Amino Acid Sequence

A set of cloned plasmid DNAs were isolated from a cDNA library (secondary culture CEF), mapped by restriction endonuclease digestion, and characterized by DNA sequence analysis (see Materials and Methods and Fig. 1). DNA sequence analysis demonstrated that the three clones indicated in Fig. 1 A contained overlapping DNA sequences. The strategy that resulted in the elucidation of the entire DNA sequence is shown in Fig. 1 B, and summarizes the minimal data required to establish the complete DNA sequence. Additional sequence data were generated for confirmation of the proposed final DNA sequence.

The nucleotide sequence obtained is summarized in Fig. 2. The DNA sequence has an open reading frame of 3,774 bases that begins with an ATG translation initiation codon at nucleotide 663. This DNA sequence encodes an amino acid sequence of 1,257 residues with a computed mass of 139,594. The computed mass is in general agreement with the estimated molecular weight, based on SDS-PAGE analysis, of avian gizzard (smooth muscle) (1, 17, 56, 79) MLCK and mammalian nmMLCK (8). However, the computed mass of chicken gizzard MLCK is only 107,534 (58), raising the possibility that the estimated molecular weight of fibroblast MLCK, based on SDS-PAGE, may be closer in magnitude to its computed mass than that for gizzard MLCK. The length of the fibroblast cDNA is also consistent with the estimated size of the mRNA for fibroblast MLCK (76). Overall, confirmation of the identity of the cloned DNA as encoding an MLCK is indicated by the: (a) ability to produce an MLCK activity from the cloned DNA (shown in succeeding sections of this report); (b) general agreement between the computed mass and the estimated molecular weight of nmMLCK; (c) exact agreement between the translated amino acid sequence and elucidated amino acid sequences for selected peptides from a tissue-isolated enzyme; and (d) immunoreactivity with anti-MLCK antibodies of the protein product produced by expression cloning.

A search of databases revealed that the closest match to

either the DNA or amino acid sequence of fibroblast MLCK was the partial sequences of chicken gizzard MLCK. In addition, the remainder of the DNA sequence of a cDNA encoding chicken gizzard MLCK was reported (58) while this manuscript was under review. Altogether, the differences between the chicken fibroblast MLCK cDNA sequence and that for chicken gizzard MLCK fall into four categories: (a) the complete DNA sequence of fibroblast MLCK reported here is at least 1,220 bp longer at the 5' end than that reported (58) for gizzard MLCK, resulting in a significantly shorter open reading frame for gizzard MLCK compared to fibroblast MLCK; (b) different DNA sequences between nucleotides 1,220 and 1,229 of fibroblast MLCK cDNA (corresponding to nucleotides 1-9 of the gizzard MLCK cDNA); (c) a single base pair difference at nucleotide 3,034 (G-3034 in fibroblast, A-1815 in gizzard) that results in a potential coding difference; and (d) a set of single base pair differences corresponding to the position of nucleotides 3,527, 3,653, 3,701 and 3,755 of the fibroblast cDNA sequence.

The potential importance of the differences between the cDNAs encoding fibroblast or gizzard MLCK can be partially addressed based on the results of our studies, and an analysis of the reports by Guerriero et al. (23) and Olson et al. (58). The DNA sequence of the cDNA encoding gizzard MLCK (23, 58) appears to be significantly shorter in its open reading frame, and there is a major divergence in the DNA sequence identity between the gizzard and fibroblast cDNAs in the first 9 bp of the noncoding region of the gizzard MLCK cDNA. A DNA sequence corresponding to nucleotides 1-9 of the gizzard cDNA is not found in the fibroblast cDNA. The DNA sequence in this region of the fibroblast cDNA was established by overlapping sequence analysis of both strands, twice on each strand, diminishing the probability of a technical artifact in the sequence analysis. Although current studies of the genomic DNA sequence should provide insight into the biosynthetic basis of this major difference between fibroblast and gizzard MLCKs, it is reasonable to speculate that the two cDNAs might be derived from the same gene by either alternative splicing or promotion. This type of biosynthetic relationship could produce in smooth muscle tissue an MLCK that is shorter in length than nmMLCK, has extensive DNA sequence identity with nmMLCK, and has a major divergence from the nmMLCK cDNA in the 5' noncoding region of the cDNA.

The single base pair difference between the fibroblast and gizzard cDNA sequences at the position corresponding to G-3034 of the fibroblast cDNA would result in a coding difference between the two MLCKs (arginine in fibroblast MLCK and glutamine in gizzard MLCK). No protein sequence analysis data for either MLCK is available for this region. However, the sequence of the fibroblast cDNA determined as part of this report was characterized from five different sequence reactions on three different cloned DNA inserts purified from two different fibroblast cDNA libraries. The DNA sequence of the gizzard cDNA (23, 58) was elucidated from two different clones isolated from two different gizzard libraries. Thus, this difference in DNA sequence, like that for the 5' ends of both cDNAs, needs to be investigated further.

The differences in DNA sequence between the fibroblast and gizzard cDNAs at positions corresponding to nucleotides 3,527, 3,653, 3,701, and 3,755 of the fibroblast cDNA

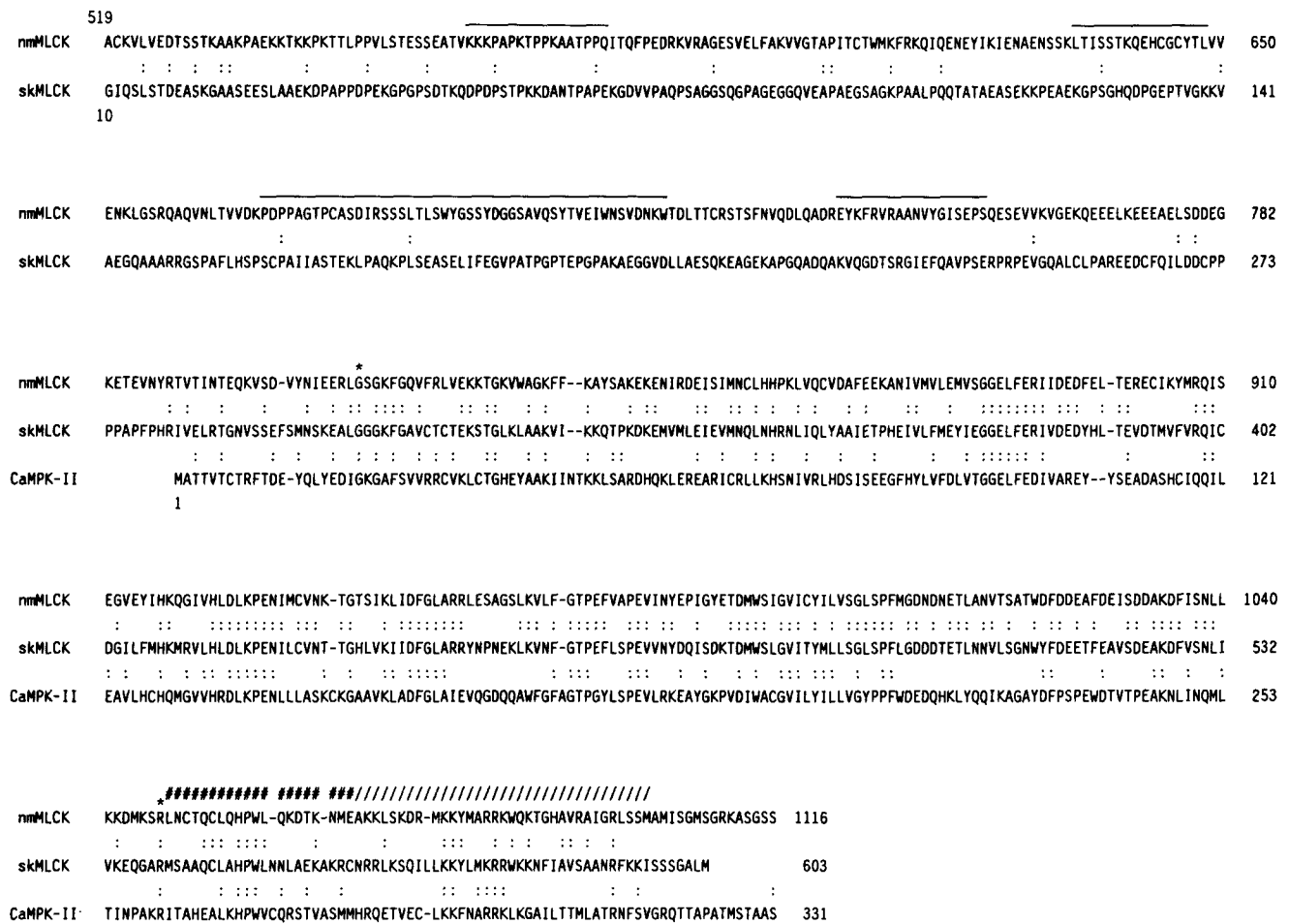
appear to correlate with a given sample of cloned DNA, based on the limited data available. Briefly, we determined the DNA sequence of this region from multiple DNA samples purified from two different fibroblast cDNA libraries and one gizzard cDNA library. Interestingly, in all cases the DNA sequence at these positions was either C or T, and all of the differences found would be silent in terms of coding potential. Further investigation of this observation was not done as part of this study, and the DNA sequence given in Fig. 2 is that obtained for the clones shown in Fig. 1 A. At this time, the presence of a C or T at these positions for either the fibroblast or gizzard cDNAs must be considered equivocal and possibly dependent on the molecular cloning history of the insert.

In contrast to the extensive stretches of DNA and amino acid sequence identity between smMLCK and nmMLCK, there is only a 29% identity between the cDNAs encoding nmMLCK and skMLCK. Overall, the database and literature searches revealed that the nmMLCK is the longest polypeptide chain for a CaM-regulated protein kinase that has been characterized, including the closely related smMLCK, and is twice the length of the skMLCK.

The amino acid sequence of nmMLCK can be divided arbitrarily into segments based on sequence similarity to other protein kinases and to other CaM regulated protein kinases (Fig. 3). For example, all of the conserved residues noted by Hanks et al. (24) in their analysis of protein kinases are contained in the nmMLCK sequence. In addition, there are other segments that have extensive amino acid sequence similarity with other CaM-regulated protein kinases (Fig. 3). Studies implicating specific segments in catalytic and regulatory function have been done (this report). The functional significance of amino acids 1,048-1,067, another region of sequence similarity noted in Fig. 3, is not known. However, the occurrence between potential catalytic and regulatory segments, and apparent conservation among certain regulated protein kinases, raised the possibility of functional importance. The COOH-terminal segment (Fig. 2) might be identical in amino acid sequence to a protein called KRP or telokin, that can be isolated from homogenates of chicken gizzard tissue (Shattuck, R. L., and D. M. Watterson, unpublished data; 40), although it has not been shown yet that KRP/telokin is a unique transcript rather than a proteolytic fragment of smMLCK.

### *Sequence Motifs Outside of the Catalytic and CaM Regulatory Segments*

The amino acid sequences of nmMLCK and smMLCK diverge significantly from skMLCK and other CaM regulated protein kinases in the amino terminal tail and the COOH terminal segments. These segments of nmMLCK have two sequence motifs that are found in receptors, and the tail segment has a third repeated motif that appears to be specific to nmMLCK and smMLCK (Fig. 4). Benian et al. (6) noted the presence of sequence motifs I and II in neural adhesion molecules (NCAMs), twitchin (a protein encoded by a *Caenorhabditis elegans* gene that is altered in motility mutants), the receptor for PDGF, and the partial amino acid sequence of gizzard MLCK. Based on the characteristics of the proteins in which these motifs are found, Benian et al. (6) raised the possibility that these motifs might be involved in subcel-



**Figure 3.** Extended amino acid sequence identities between nmMLCK and skMLCK are limited to catalytic and CaM regulatory regions. Comparison of the two MLCKs (this report and reference 73) and beta subunit of CaMPK-II (7) demonstrates that the extent of amino acid sequence identity between nmMLCK and skMLCK is characteristic of CaM-regulated protein kinases. The colon indicates positions of amino acid sequence identity with nmMLCK. The numerals indicate amino acid residues for each kinase. The asterisk marks the first and last conserved amino acids in the catalytic region of protein kinases as defined by Hanks et al. (24). The diagonal symbols denote the approximate boundaries of the calmodulin regulatory region, which includes the AM13/RS20 regions (see Fig. 5). The pound symbols denote a conserved region found in CaM-regulated and other regulated protein kinases. nmMLCK and smMLCK have sequence motifs in the tail segment that are similar to those found in receptor proteins, and have a unique motif (Motif III) that is repeated (see Fig. 4). The overlining indicates the position of motifs in this region. The portion of nmMLCK shown here is included in rMLCK1 (Fig. 5).

lular targeting or folding of the proteins. The location of these sequence motifs, called motif I and II, in the fibroblast MLCK sequence are shown in Fig. 4 along with a comparison to a similar sequence from NCAM or PDGF receptor. For ease of comparison these have been divided into motifs Ia, Ib, IIa, and IIb.

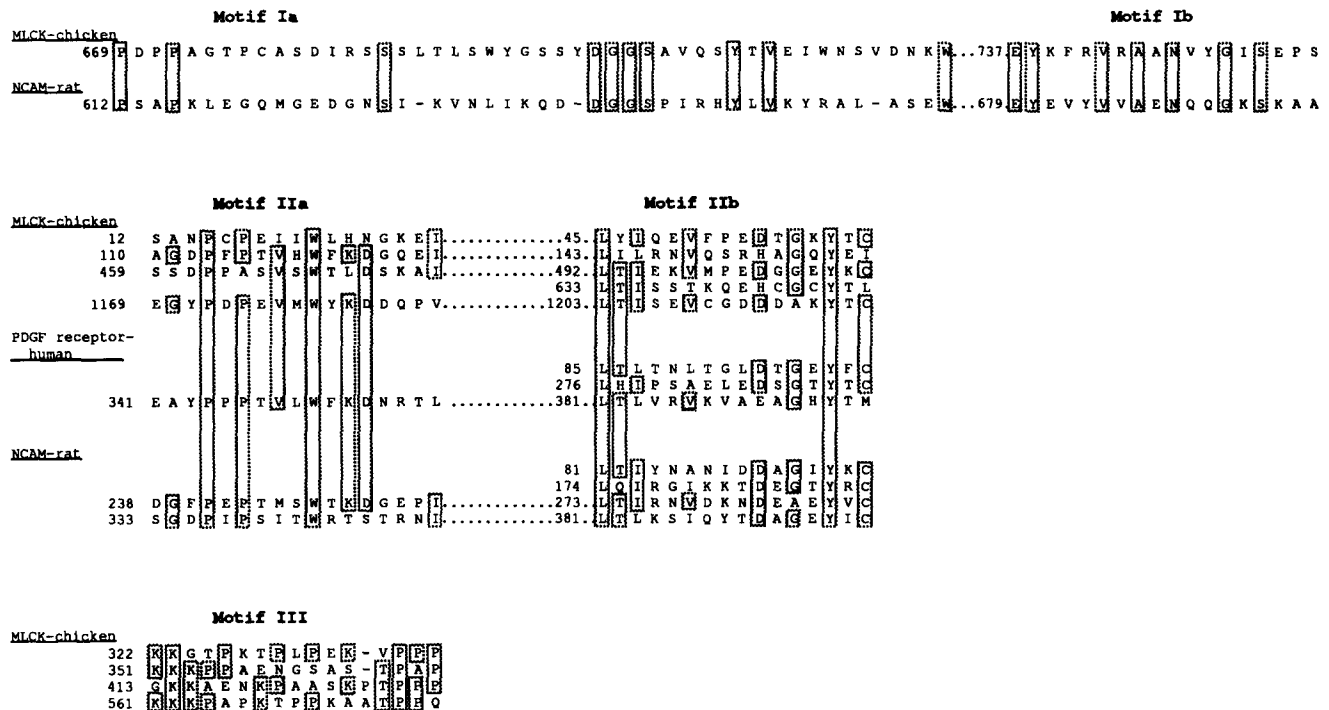
Amino acid sequence motif III, which we detected during the analysis of the fibroblast MLCK sequence (Fig. 4), occurs four times in the tail segment. At this time, this repeated motif pattern appears to be unique to nmMLCK and smMLCK. Database searches based upon motif III did not yield other proteins with this specific arrangement. However, similarity searches with a region of fibroblast MLCK (residues 300-500) that contains motif III sequences, found proteins with proline-rich regions (e.g., histone H1, neurofilament triplet H protein, and the Abl transforming tyrosine kinase). Currently, the functional significance of motifs I, II, and III in MLCK or other proteins is not known. However, based upon their relative positions within MLCK and the

types of other proteins in which they are found, the suggestion (6) that they may be involved in subcellular targeting is reasonable. Relatedly, these motifs might be key in the folding of a common type of protein structural domain.

MLCK has potential N-glycosylation sequences (e.g., residues 358, 629, 662, and 1,200), but there has been no evidence to date to indicate that MLCK is glycosylated. Computer algorithms (see Material and Methods) also reveal a number of potential phosphorylation sites (Table I). Altogether, there is the potential of fibroblast MLCK being a substrate for at least six different protein kinases.

### *rMLCK1 Is Indistinguishable from Tissue-isolated MLCK in its Kinetic and Regulatory Properties*

The catalytic and CaM regulatory segments of fibroblast MLCK, tentatively identified by sequence comparison (Fig. 3), were correlated with CaM-regulated catalytic activity (Fig. 6 and Table II) by the production of a protein, rMLCK1 (sequence data are available from EMBL/GenBank/DDJB



**Figure 4.** Sequence motifs found in membrane and functionally unrelated proteins are present in the tail and KRP-like segments of fibroblast MLCK. Previously identified (6) amino acid sequence motifs (I and II) are present in fibroblast MLCK. In addition, a new motif (motif III) has been identified. Motifs I and II from fibroblast MLCK are compared to examples from neuron cell adhesion molecule (NCAM; 71), and the PDGF receptor (22). Amino acid sequences of NCAM and PDGF receptor are from the PIR protein sequence database entries S00846 and A28206, respectively. Boxes indicate positions where the same amino acid is present in at least 50% of the sequences. Numerals indicate amino acid residue number in the sequence.

under accession number X52877), that includes amino acid residues 519–1,116 (Figs. 3 and 5). rMLCK1 is the same net length as skMLCK and includes a set of nmMLCK motifs from the tail segment. rMLCK1 was made by expression cloning in *E. coli*, and was purified through a CaM-Sepharose chromatography step. Like the MLCK activity purified from chicken gizzard, rMLCK1 has no detectable activity under standard assay conditions in the absence of CaM, and requires the presence of the calcium/CaM complex for activity. rMLCK1 is also similar to chicken gizzard MLCK in the concentration of CaM required for half-maximal stimulation. Thus, rMLCK1 includes the catalytic, autoinhibitory, and CaM recognition functions of fibroblast MLCK.

rMLCK1 responds to mutations in CaM similar to tissue-isolated MLCK (Fig. 6 and Table II). Point mutations at E84 and E120 of CaM result in an increase in the apparent  $K_m$  of the CaM/MLCK and CaM/rMLCK1 complex for peptide substrate (Table II). Although apparent effects on  $V_{max}$  were also noted, the consistent trend with the CaM mutations is to increase the apparent  $K_m$  of the complex. The peptide substrate specificity of rMLCK1 also resembles that of gizzard MLCK. When peptide substrates for other protein kinases such as CaMPK-II or cAMP-dependent protein kinase were tested at concentrations similar to that of the light chain peptide substrate (50  $\mu$ M), rMLCK1 had <10% of the phosphotransferase activity it had with the myosin light chain peptide substrate (data not shown). Therefore, rMLCK1 has the kinetic properties, peptide substrate specificity, and CaM recognition properties of an MLCK isolated from a vertebrate tissue.

### Design, Production, and Analysis of a Suppressor Mutant

Results from studies that used peptide analogues of residues 1,082–1,101 in the MLCK sequence (48, 50), site-specific mutagenesis, and computational chemistry analyses of CaM (14, 80; Table II), and enzyme kinetic studies of CaM/MLCK complexes (Table II) raised the possibility of complementary charge features within CaM and MLCK that are important in the formation and functioning of CaM/MLCK signal transduction complexes. The potential importance of protein charge, or electrostatic, properties in biological macromolecular recognition has been recognized for some time (59), and the specific data that showed (14, 80) that charge reversal mutations of CaM alter its ability to interact with MLCK are consistent with this possibility. If there is a complementary charge component to CaM/MLCK recognition and function, then it might be possible to engineer a suppressor mutant of MLCK by using charge reversal mutagenesis of MLCK between residues 1,082–1,101. A suppressor mutation is one in which a mutation of one member (e.g., MLCK) of a pair of interacting proteins (e.g., CaM and MLCK) can compensate for, or suppress, the effects of a mutation in the other component (e.g., CaM).

As a direct test of this chemical complementarity hypothesis we changed the positively charged Arg-Arg-Lys sequence in MLCK (residues 1,082–1,084) to a Glu-Glu-Glu negatively charged cluster, and tested the ability of this mutant MLCK to suppress the functional effects of Glu to Lys changes in CaM. The resultant enzyme, rMLCK2 (Fig. 5),



**Table I. Potential Sites in Nonmuscle and Smooth Muscle Myosin Light Chain Kinases for Phosphorylation by Other Protein Kinases\***

cAMP/cGMP kinases	CaMPK-II	Protein kinase C <sup>‡</sup>	Casein kinase II	Tyrosine kinase	cdc2 kinase
Ser-219	Thr-96	Thr-55	Thr-76	Thr-46	Thr-568
Ser-269	Ser-179	Ser-249	Thr-130	Thr-621	
Thr-325	Thr-242	Ser-269	Ser-181		
Ser-1072	Thr-246	Thr-325	Ser-194		
Ser-1100§	Ser-372	Thr-438	Ser-218		
Ser-1113§	Ser-460	Ser-477	Ser-272		
	Ser-685	Ser-529	Ser-274		
	Ser-726	Thr-540	Thr-300		
	Thr-793	Thr-559	Ser-302		
	Ser-812	Ser-630	Ser-436		
	Ser-910	Ser-637	Thr-638		
	Ser-954	Thr-721	Thr-664		
	Ser-1100§	Ser-812	Ser-693		
	Ser-1113	Ser-826	Ser-778		
		Ser-839	Ser-839		
		Thr-898	Ser-882		
		Ser-940	Thr-898		
		Ser-957	Thr-1150		
		Ser-1108	Thr-1204		
		Thr-1217	Ser-1223		

\* Detected using the PROSITE program (See Materials and Methods) except as noted. Residue numbers are those of nmMLCK (this report). CaMPK-II (CaM-dependent protein kinase II) sites are based on the consensus sequence Arg-X-Y-Ser/Thr (69), while the cdc2 kinase site was located with the consensus, Ser/Thr-Pro-X-Lys (63).

<sup>‡</sup> Protein kinase C phosphorylates smMLCK (57), but sites have not been identified.

<sup>§</sup> Indicates that a particular site has been verified experimentally (28, 48, 60, 61) in a smooth or nonmuscle MLCK. Ser-1100 in MLCK was not detected by PROSITE.

was able to suppress the kinetic effects of charge reversal mutations in CaM in which glutamic acid residues were changed to lysines (Table II). rMLCK2 remained CaM dependent, indicating that the suppression is not due to a loss of intramolecular inhibition. As summarized in Table II, the trend of an increased apparent  $K_m$  for peptide substrate by enzyme complexes that contain mutant CaMs and wild-type protein kinases is reversed toward normal when the complex contained rMLCK2. An equivalent suppressor effect was not seen with other charge reversal mutations in this sequence (e.g., rMLCK3, rMLCK4, or rMLCK5), indicating that the full suppressor effect requires the cluster charge reversal.

### A Chimeric nmMLCK/skMLCK Enzyme Is Isofunctional with nmMLCK

The ability of mutations in the RS20 region of MLCK to suppress the functional effects of CaM mutations (rMLCK2; Fig. 5), combined with the previous demonstration (48, 50) that fragments and peptide analogues of this sequence have selective high affinity CaM binding activity, provides a more formalized proof that this region of MLCK is quantitatively important in CaM recognition. To explore further the molecular basis of the selective recognition of CaM by MLCK and test for functional differences in CaM recognition and regulation between the nmMLCK and skMLCKs, we produced and characterized a chimeric enzyme in which residues 1,081-1,099 of the rMLCK1 were replaced by the corresponding sequence from skMLCK (Fig. 5). The enzyme,

**Table II. Analysis of a MLCK Suppressor Mutant Produced by Site-specific Mutagenesis of the CaM Recognition Sequence\***

CaM/MLCK complex				
Protein kinase	Calmodulin <sup>‡</sup>	Peptide substrate $K_m$ <sup>§</sup>	Ratio <sup>  </sup>	
		$\mu M$		
MLCK (wild type)	+	W.T.	10.6 ± 2.5 (11)	1.0
		E84K mutant	40.8 ± 6.0 (3)	3.8
MLCK (wild type)		E120K mutant	65.2 ± 10.5 (3)	6.2
		W.T.	11.1 ± 2.8 (13)	1.0
rMLCK1 (wild type)	+	E84K mutant	47.5 ± 6.6 (8)	4.3
		E120K mutant	85.6 ± 17.4 (9)	7.7
rMLCK2 (suppressor mutant)	+	W.T.	10.9 ± 3.7 (10)	1.0
		E84K mutant	16.0 ± 5.3 (7)	1.5
		E120K mutant	18.3 ± 4.0 (6)	1.7

\* rMLCK2 differs from rMLCK1 only at the three charge reversal mutation sites (see Fig. 5), yet it is able to suppress the functional effects of CaM mutations (compare ratios of complexes containing wild-type or mutant CaMs). MLCK, rMLCK1, and rMLCK2 have no detectable activity in the absence of calcium and CaM under the assay conditions used.

<sup>‡</sup> Wild-type (W.T.) calmodulin refers to either tissue-isolated vertebrate calmodulin, recombinant DNA encoded and produced vertebrate calmodulin, or VU-1 (SYNCAM) calmodulin. These are functionally equivalent under the conditions used. The mutant calmodulins differ from SYNCAM calmodulin only at the single sites noted (E84 and E120). At least a fourfold excess of calmodulin (0.2-3  $\mu M$ ) over the concentration required to achieve maximal activation was used in these determinations.

<sup>§</sup> The values represent the average apparent  $K_m$  ± the standard deviation for the number, in parentheses, of experiments (see Materials and Methods).

$$\text{|| Ratio} = \frac{K_m \text{ of protein kinase activated by a given calmodulin}}{K_m \text{ of same protein kinase activated by wild-type calmodulin}}$$

The ratio of the  $K_m$ 's of a protein kinase activated by wild-type calmodulin is 1.0. An increase in the ratio indicates the decreased ability of a given calmodulin to activate that protein kinase.

termed rMLCK6, was produced in *E. coli*, purified through CaM-Sepharose chromatography, and characterized in terms of its responses to wild-type and mutant CaMs (Fig. 6).

rMLCK6 is functionally indistinguishable from rMLCK1. rMLCK6 requires CaM for activity, thereby demonstrating that the autoinhibition activity has not been disturbed. The concentration of CaM required for half-maximal stimulation of rMLCK6 is similar to that of rMLCK1, as is the response to the E84K and E120K mutant CaMs (Fig. 6). These data, combined with the results of the following sections, demonstrate that more than one amino acid sequence can produce the same CaM recognition properties within the context of an active, CaM-dependent enzyme, and suggest a consensus CaM recognition sequence for MLCKs. As a caveat, it should be noted that the results do not mean that the secondary and tertiary structures are equivalent; the results do raise the possibility that more than one structure can accomplish the same function.

### CaMPK-II CaM Binding Sequences in MLCK Convert the CaM Recognition Properties of MLCK toward Those of CaMPK-II

Previous studies have shown (80) that CaMPK-II is less sensitive than MLCK to the E84K mutation in CaM. CaMPK-II also has a region of amino acid sequence with a high degree of similarity to the RS20 region of MLCK (Fig. 3). Like MLCK, peptide analogue studies of CaMPK-II sequences

ACTIVITY				AMINO ACID SEQUENCES IN CaM REGULATORY SEGMENT			
CaM BINDING		KINASE DEPENDENCE		AM13 REGION		RS20 REGION	
<b>CHARGE REVERSAL AND SUPPRESSOR MUTATIONS WITHIN CaM RECOGNITION REGION</b>							
rMLCK1	+	+	+	1065	N M E A K K L S K D R M K K Y M A	1082	R R K W Q K T G H A V R A I G R L S S M A
rMLCK2	+	+	+				E E E
rMLCK3	+	+	+				E
rMLCK4	+	+	+				E
rMLCK5	+	+	+				E E
<b>CHIMERIC MUTATIONS WITHIN CaM RECOGNITION REGION</b>							
rMLCK1	+	+	+	1065	N M E A K K L S K D R M K K Y M A	1082	R R K W Q K T G H A V R A I G R L S S M A
rMLCK6	+	+	+				M K R K N F I S A N F K
rMLCK7	+	+	+				L K G A I L T T M L A T N F
rMLCK8	+	+	+				G
rMLCK9	+	+	+				L
rMLCK10	+	+	+				T
rMLCK11	+	+	+				T
rMLCK12	+	+	+				T T M
<b>SEQUENCE INVERSION AND RANDOM MUTATIONS WITHIN CaM RECOGNITION REGION</b>							
rMLCK1	+	+	+	1065	N M E A K K L S K D R M K K Y M A	1082	R R K W Q K T G H A V R A I G R L S S M A
rMLCK13	+	+	+				L G I A R V A G T K Q W K R
rMLCK14	+	-	-				E P M L S E Q L V D S R A W Q /
rMLCK15	+	-	-				E S F R R P M L S E Q L V D S R P W Q /
<b>DELETION OF AUTOINHIBITORY AND CaM RECOGNITION REGIONS</b>							
rMLCK1	+	+	+	1065	N M E A K K L S K D R M K K Y M A	1082	R R K W Q K T G H A V R A I G R L S S M A
rMLCK16	-	+	-		N M E /		
<b>CHARGE MUTATIONS WITHIN AUTOINHIBITORY REGION</b>							
rMLCK1	+	+	+	1065	N M E A K K L S K D R M K K Y M A	1082	R R K W Q K T G H A V R A I G R L S S M A
rMLCK17	+	+	-		E E E E E E		
rMLCK18	+	+	+				E
rMLCK19	+	+	+		E		
rMLCK20	+	+	+			E	E
rMLCK21	+	+	+		D		

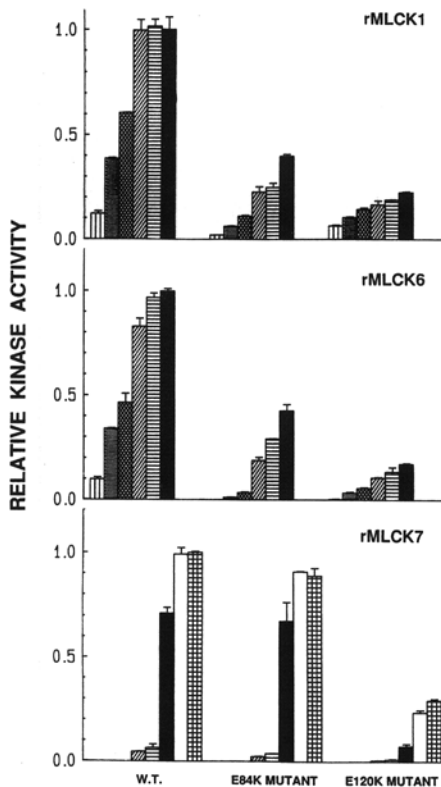
Figure 5. Summary of the primary structure and functional properties of the 20 mutant MLCKs used in this study. The ability (+) or inability (-) of each protein kinase either to bind CaM, phosphorylate peptide substrate, or be activated by CaM is indicated. The portion of the fibroblast MLCK sequence shown in Fig. 3 encodes a CaM-dependent MLCK activity (rMLCK1). This sequence contains the catalytic and proposed CaM regulatory segments as well as a set of tail segment motifs identified in Fig. 4. Shown here is a comparison of the amino acid sequences within the CaM regulatory segment (AM13/autoinhibitory region and RS20/CaM recognition region) of the 20 MLCK mutants to that of the parent rMLCK1; only the differences in amino acid sequence between the mutants and rMLCK1 are shown. For rMLCK14, 15, and 16 the diagonal symbol indicates the carboxy terminus of these proteins.

have implicated this region of CaMPK-II in CaM binding (25). Therefore, as an attempt to gain additional insight into the molecular basis of differential CaM recognition by protein kinases (80), we made and characterized a chimeric enzyme in which a portion of the RS20 region of MLCK was replaced by the corresponding sequence from CaMPK-II.

The chimeric enzyme, termed rMLCK7 (Fig. 5), responded (Fig. 6) to wild-type and mutant CaMs similar to CaMPK-II, but the autoinhibition, substrate recognition, and phosphotransferase activities of MLCK were maintained. As with CaMPK-II isolated from vertebrate tissue (80), the concentration of CaM required for half-maximal activation of rMLCK7 is greater than that seen with MLCK or rMLCK1. Similarly, the comparative lack of sensitivity of rMLCK7 to the E84K CaM mutation is reminiscent of that seen (80) with tissue-isolated CaMPK-II. Thus, there is a partial suppressor effect of substituting the CaMPK-II sequence for the MLCK sequence in the RS20 region. A series of point and cluster mutations in which the MLCK consensus sequence was

converted to the CaMPK-II sequence in this region resulted in an increase in the concentration of CaM required for half-maximal activity like that seen with CaMPK-II and the MLCK/CaMPK-II chimeric enzyme (rMLCK7). However, the partial suppressor effect seen with CaMPK-II and rMLCK7 was not seen with partial replacements, such as the changes found in rMLCK8, rMLCK9, rMLCK10, rMLCK11, and rMLCK12 (Fig. 5). This indicates that the differential response of rMLCK7 to mutant CaMs may be a feature of some combination of the several sequence differences from the consensus MLCK sequence. This possibility was not investigated further as part of this study.

Overall, the ability to generate, by altering the RS20 region of MLCK, enzymes that maintain their substrate specificity but respond differentially to wild-type and mutant CaMs supports the hypothesis (48) that the RS20 region of nmMLCK and smMLCK is quantitatively important in CaM recognition. The MLCK mutants described in this and the preceding sections are like smMLCK in their lack of



**Figure 6.** The CaM recognition features of fibroblast MLCK can be converted to those of CaMPK II by changing the RS20 region. The CaM binding region, RS20, of rMLCK1 was replaced with the corresponding sequences from skMLCK (rMLCK6) or CaMPK-II (rMLCK7). The resultant amino acid sequences are shown in Fig. 5. Shown here is the dose-dependent activation of each protein kinase by wild type (W.T.) or mutant CaMs. The mutant CaMs contain single Glu to Lys substitutions (E84K or E120K). The ordinate refers to the activity of each kinase on a 0–1.0 scale, where 1.0 is the maximum activity obtained with wild-type CaM, for each protein kinase. Error bars indicate the range of duplicate determinations. Each CaM concentration is represented by a bar: 3 (vertically striped), 6 (speckled), 10 (crosshatched), 30 (diagonal), 60 (horizontal), 260 (solid), 1,200 nM (open), and 8,000 nM (windownpane).

significant activity with caltractin (data not shown), a naturally occurring CaM analogue (36). This demonstrates that the gross recognition features that allow discrimination between CaM and closely related analogues found in the same cell are maintained in these mutant MLCK, even when there are changes in response to wild-type and mutant CaMs.

#### ***Inversion of the Sequence within the CaM Binding Region Results in a Protein That Is Still CaM Dependent and Not Constitutively Active***

All of the mutant MLCKs described in the preceding sections are CaM dependent, i.e., they are not constitutively active. This is noteworthy because altogether the mutations (see Fig. 5) involved changing most of the amino acids that have been implicated in some autoinhibition models, and all of the critical amino acids of the pseudosubstrate hypothesis (61) have been altered. This raises concerns about some of the basic assumptions of the autoinhibition models in which the autoinhibitory sequences of MLCKs have been equated with the CaM binding sequences. Therefore, we produced a

mutant MLCK (rMLCK13) in which the sequence within this region was inverted (Fig. 5) as part of an attempt to further explore the basic assumptions of autoinhibition models, as well as gain further insight into the features of the RS20 region that are required for binding and activation of MLCK.

rMLCK13 has the same amino acid composition, hydrophobic moment, and average hydrophobicity as rMLCK1 (Fig. 7 top), although the amino acid sequence within the RS20 region is inverted (Fig. 5). The proposed amphiphilic nature of the sequence is also maintained in the inverted sequence (Fig. 7 top). rMLCK13 is not constitutively active and can be activated by calcium and CaM, although there is an increase in the concentration of CaM required for half-maximal activity (Fig. 7 bottom), which is reminiscent of the MLCK/CaMPK-II chimeric enzyme (rMLCK7). However, the response of rMLCK13 to mutant CaMs (Fig. 7 bottom) is much more diminished than that seen with rMLCK7 (Fig. 6). rMLCK13 still has the ability to discriminate between CaM and closely related proteins as evidenced by the inability to detect significant activity (>10% of background) when caltractin is used in the assays instead of CaM.

These data demonstrate that the orientation of the amino acid sequence, relative to the catalytic segment, is not a critical feature of MLCK for proper recognition of CaM and relief of intramolecular inhibition, i.e., activation. The computed average hydrophobicity and hydrophobic moment of the sequences between residues 1,082–1,098 for the mutant MLCKs described in this and preceding sections also demonstrates that proper recognition and functionally coupled binding of CaM does not have a simple relationship with these features, at least when function is assayed in the context of an active CaM-dependent enzyme. Overall, the fact that extensive alterations of the sequence and the general features of the RS20 region does not produce a constitutively active, CaM-independent enzyme suggests that the autoinhibitory activity of MLCK may not be as critically dependent on the amino acid residues in the RS20 region as previously assumed based on model peptide studies.

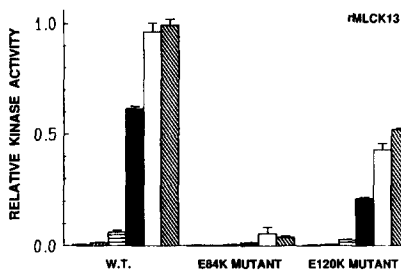
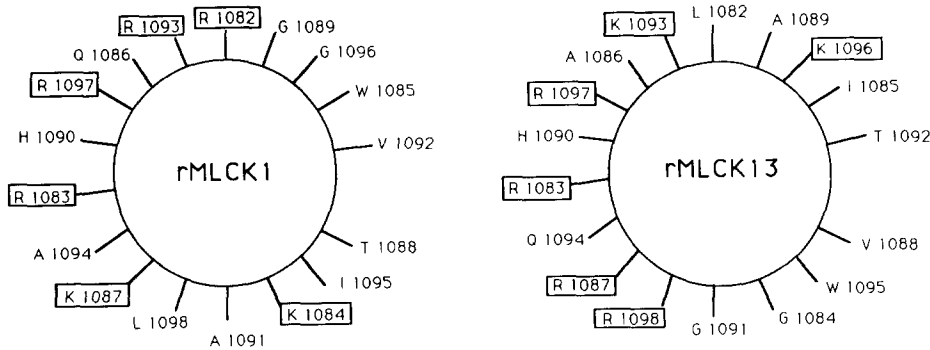
#### ***CaM Binding by MLCK Is Necessary but Not Sufficient for Enzyme Activation***

All of the mutant enzymes described in the preceding sections can be activated by CaM and calcium. However, rMLCK14 and rMLCK15 (Fig. 5), generated by random mutagenesis of the RS20 region, bind CaM as judged by interaction with CaM-Sepharose columns and by gel overlay assays (Fig. 8), but cannot be activated by the calcium/CaM complex under these conditions.

Altogether, the data for rMLCKs 1–15 demonstrate that the sequence requirements for CaM binding are less strict than those for CaM binding with resultant activation. Thus, while mutations in the RS20 region have not resulted in a constitutively active enzyme, it is possible for mutations in this region to functionally uncouple CaM binding from relief of autoinhibition.

#### ***Removal of the AM13 and RS20 Regions Results in Constitutive Activity with Maintenance of Substrate Specificity***

The ability to alter extensively the amino acid sequence of the RS20 region without generating a CaM-independent and



for the average of two experiments. The CaM concentrations indicated by the bars are 10 (crosshatched), 30 (left-directed diagonals), 60 (horizontal), 260 (solid), 1,200 (open), and 4,000 nM (right-directed diagonals).

**Figure 7.** CaM-dependent MLCK activity is maintained despite inversion of the CaM recognition region. (Top) The helical wheel projections for rMLCK1 and rMLCK13 demonstrate that the potential amphiphilic nature is maintained, but the relative orientation of the positively charged surface is altered. Numbers refer to the relative positions of these amino acid residues in nmMLCK (Fig 2). rMLCK13 differs from rMLCK1 by the inversion of the amino acid sequence between residues 1,082 and 1,098 (see Fig. 5). However, the hydrophobic moments (0.62) and average hydrophobicity (-0.47) are identical. Boxed residues denote charged amino acids. (Bottom) The dose-dependent activation of rMLCK13 by wild type (WT) and mutant CaMs was tested as described in Materials and Methods. The mutant CaMs and relative kinase activity calculations are the same as described in Fig. 6. Error bars indicate the range of values

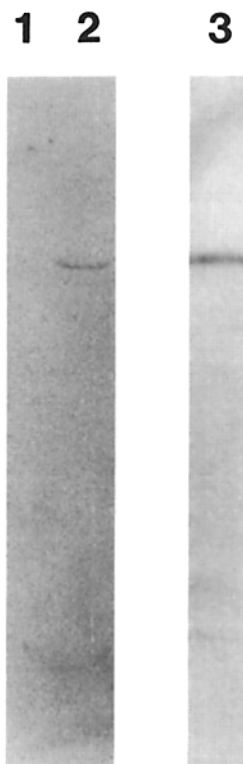
constitutively active enzyme required a reexamination of autoinhibition, and prompted an attempt to define more exactly the boundaries of the CaM regulatory segment. Previous studies (50) showed that a synthetic peptide analogue of the AM13 region (residues 1,068-1,080) was a competitive inhibitor of MLCK activity, but did not have the high affinity CaM binding activity of RS20 analogues. Therefore, as an initial step we truncated rMLCK1 immediately preceding the AM13 region by changing the codon for Ala-1068 to a translation termination codon. When this construct was expressed in *E. coli*, a constitutively active enzyme (rMLCK16) was produced (Fig. 9). rMLCK16 did not bind to CaM-Sepharose columns and did not bind CaM in gel overlay assays.

Although rMLCK16 has lost its ability to be autoinhibited and modulated by CaM, it has maintained substrate specificity. The apparent  $K_m$  for the peptide substrate is  $\sim 10 \mu\text{M}$ , similar to that for rMLCK1 or smMLCK (Table II) in the presence of CaM. These results demonstrate that autoinhibition and CaM dependence of activation require the presence of the AM13 and RS20 regions, but MLCK catalytic activity does not require the presence of these regions. Based on these results, rMLCK16 is an excellent candidate for transfection studies that seek to overexpress a constitutively active protein kinase with the strict substrate recognition properties of an MLCK.

#### Mutation of the AM13 Region Produces a Constitutively Active Enzyme That Binds CaM

Overall, the results of the preceding sections demonstrate: (a) the importance of the AM13 and RS20 regions of MLCK in order to have an autoinhibited enzyme that is activated selectively by CaM; (b) the requirement for a consensus se-

quence in the RS20 region for proper CaM recognition and activation; and (c) the potential sensitivity of autoinhibition activity to the amino acid sequences in the AM13 region. However, the suggested involvement of the AM13 region in



**Figure 8.** CaM binding by MLCK is necessary but not sufficient for enzyme activation. rMLCK14 was produced by random mutagenesis of the RS20 region, binds CaM by two criteria, and cannot be activated by CaM under the conditions used. Lanes 1 and 2 are Western blots with anti-MLCK antibodies that demonstrate the binding of rMLCK14 to CaM-Sepharose in a calcium-dependent manner, and lane 3 demonstrates CaM binding by a gel overlay technique. Lane 1 contains proteins that did not bind to CaM-Sepharose in the presence of calcium; lane 2 contains proteins that bound in the presence of calcium and eluted with EGTA. The EGTA eluate containing rMLCK14 (lane 2) also binds  $^{125}\text{I}$ -CaM in the presence of calcium as demonstrated by gel overlay (lane 3). rMLCK14 does not bind  $^{125}\text{I}$ -CaM in the presence of EGTA (not shown).

autoinhibition is based on peptide analogue studies (50) and deletion mutagenesis results that removed more than the AM13 sequence (preceding section). Therefore, we used substitution mutagenesis to explore how a perturbation of the charge properties of the AM13 region would affect autoinhibition activity. As a starting point, we again used a cluster charge reversal mutagenesis approach (14, 80).

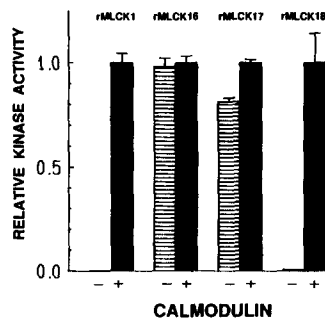
In the charge reversal mutant rMLCK17, all of the positively charged amino acid residues in the AM13 region have been changed to negatively charged glutamic acid residues (Fig. 5). As summarized in Fig. 9, rMLCK17 is constitutively active and binds CaM, but has little response to CaM. In fact, the ratio of activity for rMLCK17 in the presence or absence of CaM is similar to that observed with rMLCK16, which is a constitutively active deletion mutant. The ability of the cluster mutation in the AM13 region to generate a constitutively active enzyme, however, was not observed in selected point charge reversals in this region, e.g., rMLCK18 and rMLCK19 of Figs. 5 and 9. Similarly, combinations of selected point charge reversals in the AM13 region with point charge reversals in the RS20 region (e.g., rMLCK20 of Fig. 5) did not result in a constitutively active enzyme or a suppressor mutant (data not shown). The ability of various multiple combinations of point charge reversals in the AM13 region to produce constitutive activity was not tested as part of the study reported here.

Altogether, these results and those from the preceding sections demonstrate that the AM13 region is more important in autoinhibition than the RS20 region when the sequences are found in the context of an active, CaM-dependent enzyme. Therefore, the CaM regulatory unit in a functional kinase would include, as a minimum, both the AM13 and RS20 regions. Overall, the results suggest a segmental organization of functional units within MLCK that is more similar to CaMPK-II than previously thought.

The topological similarities in the organization of the CaM regulatory segments of MLCK and CaMPK-II raised the possibility that their functional mechanisms could be similar. It has been shown that autophosphorylation of Thr-286/287 during the activation of CaMPK-II results in a constitutively active enzyme (69). Further, when Thr-286/287 in CaMPK-II was changed to an Asp, CaMPK-II became constitutively active. Although autophosphorylation of MLCK has not been demonstrated, Thr-286/287 of CaMPK-II aligns with Ser 1072 in fibroblast MLCK (Fig. 3), and Ser-1072 is included in the autoinhibitory AM13 region of MLCK. To explore further the possible similarities in mechanism of activation for these two CaM regulated enzymes, Ser-1072 of MLCK was changed to Asp (production of rMLCK21, Fig. 5). However, rMLCK21 is not constitutively active and requires CaM for activity (data not shown). Therefore, the similarity in general segmental organization between MLCK and CaMPK-II cannot be extended to similarities in detailed mechanisms of activation, indicating the potential use of this mechanistic step for development of selective inhibitors.

### Summary Model and Activity

A summary model of the chicken nmMLCK is shown in Fig. 10. Noted in Fig. 10 are the relative positions of selected regions implicated in function. There are several implications of this model based on the structure-function correlates presented here. For example, the ability of MLCK to with-



**Figure 9.** Constitutively active, CaM-independent MLCK activity results from removal or perturbation of the autoinhibitory (AM13) region. Deletion of the AM13 and RS20 regions by truncation of rMLCK1 at residue 1068 (production of rMLCK16) results in a CaM-independent and constitutively active MLCK. A cluster charge reversal mutation with-

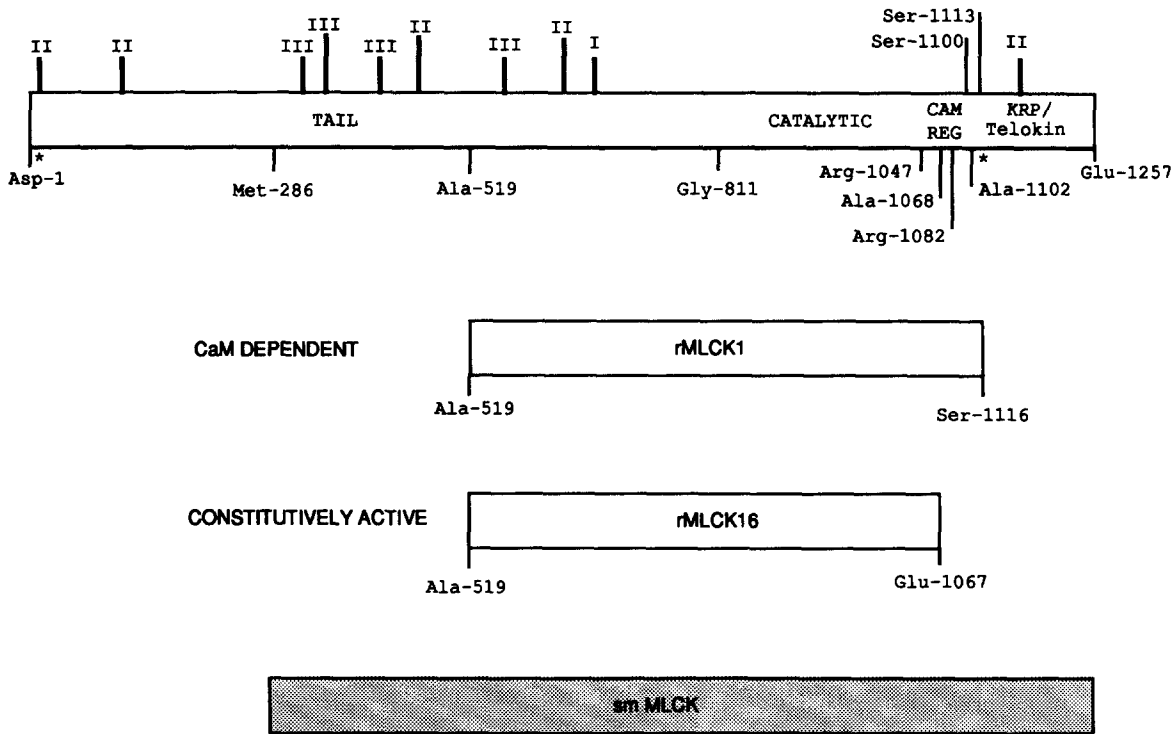
in the AM13 region (production of rMLCK17) also results in a constitutively active enzyme with little CaM dependence. The effects of the cluster charge reversal were not mimicked by the point charge reversals in this region (rMLCK18, rMLCK19, and rMLCK20). The activation of these enzymes in the absence (- under horizontal bar) or presence (+ under solid bar) of 1  $\mu$ M wild-type CaM was determined as described in Fig. 6. Error bars indicate the range of values for duplicate determinations. The changes in the amino acid sequence of rMLCK1 for these mutant MLCKs are summarized in Fig. 5.

stand a number of changes and rearrangements within the CaM recognition region suggests that mutations or individual microheterogeneities within the MLCK gene encoding this portion of MLCK may not result in drastic changes in the phenotype of an organism. This possibility, plus the apparent selectivity for CaM recognition and MLCK autoinhibition by the CaM regulatory unit, make this region appealing as a potential target site for future development of selective antagonists, complementing the targeting of the active site/catalytic segment.

The number of potential phosphorylation sites (Table I) within nmMLCK raises the possibility of significant regulatory "cross-talk" between the CaM/MLCK signal transduction pathway and several others involved in the regulation of cell proliferation and development. The ability of MLCK to withstand significant mutations within the RS20 region and to function without the presence of the KRP segment suggests the feasibility of mutating the amino acid sequence at certain phosphorylation sites, yet maintain function. Such mutant MLCKs might be useful in transfection experiments designed to probe the biological significance of phosphorylation sites identified by *in vitro* studies, e.g., phosphorylation sites at amino acids 1,100 and 1,113. Finally, the differences in the proposed lengths of nmMLCK and smMLCK allow the potential design of peptides for production of selective antibodies, or the design of selective nucleotide probes and antisense constructs.

### Treatment of CEF and 3T3 Cells with MLCK Antisense Oligodeoxynucleotides

The availability of a cDNA sequence for a nmMLCK, and recombinant DNA constructs that encode either various segments of MLCK or constitutively active MLCK activity, provide the opportunity to probe directly how MLCK and its segmental organization are related to *in vivo* activity. As a first step, we used synthetic oligodeoxynucleotides based on the cDNA sequence described here, to decrease the levels of MLCK and correlate the decrease with changes in cell morphology. Previous studies (75, 76) of MLCK levels in v-src-transformed CEF correlated a selective decrease in MLCK



**Figure 10.** Schematic model of nmMLCK segmental organization. A schematic summary of the amino acid sequence of chicken fibroblast MLCK (top line) is compared to two rMLCKs (rMLCK1 and rMLCK16) and the sequence of chicken gizzard MLCK (smMLCK). As described in the text, these four protein kinases are indistinguishable in their catalytic properties, and three of the four (fibroblast MLCK, gizzard MLCK, and rMLCK1) are indistinguishable in terms of their response to wild-type and mutant CaMs. CaM recognition of rMLCK1 can be altered by mutations in the RS20 region (Arg-1,082 to Met-1,101). rMLCK1 can also be converted to a constitutively active enzyme with retention of CaM binding and substrate recognition activities by mutations in the AM13 region (Ala-1,068 to Met-1,080). Although Gly-811 and Arg-1047 are, respectively, the first and last conserved amino acids in the catalytic region as defined by the similarity analysis of Hanks et al. (24), the shortest sequence with full activity is rMLCK16 (Ala-519 to Glu-1,067), indicating that the minimal sequence for full MLCK activity has not been defined yet. Ala-1,102 represents the postulated start of the KRP/telokin sequence (Shattuck, R. L., and D. M. Watterson, unpublished data; 40), and the sequence equivalent to smMLCK starts at Met-286. The asterisks indicate the relative positions in the open reading frame used for the design of nmMLCK-selective and common antisense oligodeoxynucleotides. The two phosphorylation sites that have been identified (see Table I) are Ser-1113 and Ser-1100. The roman numerals (I, II, III) indicate the relative location of sequence motifs (see Fig. 4) found outside the catalytic and regulatory segments.

activity, immunoreactive protein levels and mRNA levels with the altered, more rounded morphology and growth properties of virally transformed cells. However, transformed cells have a number of biochemical changes, several

of which may have a direct or indirect effect on cell morphology and components of the cytoskeleton. Therefore, we used the selective and more direct approach of antisense oligodeoxynucleotide treatment (34, 41) in order to probe further

	5' → 3'	
5' MLCK sense	663 A T G G A T G G A A G T C A A G T G A T	682
5' MLCK antisense	A T C A C T T G A C T T C C A T C C A T	
5' MLCK mismatch	A T G A C T A G A C T T G C T T C C A T	
3' MLCK sense	3966 A T G G C A A T G A T T T C T G G T A T	3985
3' MLCK antisense	A T A C C A G A A A T C A T T G C C A T	
3' MLCK mismatch	A T A C A C G A A A T A C T T G C C A T	

**Figure 11.** Synthetic oligodeoxynucleotides based on the nmMLCK cDNA sequence and used in antisense experiments. The sequences of the MLCK sense, antisense, and 4 base-mismatch oligodeoxynucleotides are shown. Two regions of the cDNA sequence (Fig. 2) were used to design the antisense constructs and controls: nucleotides 663–682 (5' MLCK) and nucleotides 3,966–3,985 (3' MLCK). As a control for each antisense, oligodeoxynucleotides corresponding to the “sense” sequence and a 4 base-mismatch antisense sequence were made. Each of the sequences were compared with known DNA sequences in Genbank and EMBL databases and no alignments with more than 16 of 20 matches were found.

the correlation of altered cell morphology and decreased MLCK levels.

As shown in Fig. 11, two regions of the cDNA sequence of CEF MLCK were used to design the 20 base antisense oligodeoxynucleotides. The 663–682 antisense construct (5'-MLCK antisense) is targeted to an area of MLCK that is unique to nmMLCK, while the 3,966–3,985 antisense construct (3'-MLCK antisense) is targeted to a region that appears to be common to nmMLCK, smMLCK and smooth muscle KRP.

Treatment of CEF or 3T3 cells with the 5' or 3' MLCK antisense oligodeoxynucleotides resulted in a striking morphological change (Fig. 12). Both CEF and 3T3 cells exhibited a more rounded morphology, reminiscent of the morphological changes seen in *v-src*-transformed cells. The morphological change was time and dose dependent, with the majority of the cells showing a shape change after 16 h in the presence of 30  $\mu$ M antisense oligodeoxynucleotide. In contrast, dose-dependent morphological changes were not seen in cells treated with the sense oligodeoxynucleotides, the mismatch oligodeoxynucleotides or solvent (water) control. In parallel experiments, treatment of cells with MLCK antisense oligodeoxynucleotides resulted in decreased levels of immunoreactive MLCK, as determined by Western blot analysis (Fig. 13).

The effects of the antisense oligodeoxynucleotides are nontoxic and reversible. As illustrated in Fig. 12, there was a restoration of cellular morphology to the normal phenotype when the antisense oligodeoxynucleotides were removed from the cultures (by changing to a cell culture medium lacking oligodeoxynucleotide and allowing the cells to proliferate for an additional 72 h). During recovery there was also an apparent increase in cell number and a return of MLCK activity to control levels. Therefore, the lack of an effect of the various control treatments, and the reversibility of the effect of the antisense oligodeoxynucleotide treatment, demonstrate that the effect on cell morphology is selective and nontoxic under these conditions. In addition to the reversible and nontoxic effect on morphology, there appears to be an effect of antisense oligodeoxynucleotides on cell proliferation. For example, the cell densities in the control cultures were  $\sim$ 2.5-fold greater than those in the antisense-treated cultures after 40 h. These results raise the possibility of a role for nmMLCK in cell proliferation as well as cell morphology and motility. Altogether, these studies provide a direct link between changes in nonmuscle cell phenotype and MLCK activity, are consistent with conclusions drawn from previous studies (75) of cells transformed by oncogenic viruses, and provide the knowledge required for a more detailed analysis of how the CaM/MLCK signal transduction pathway is coupled to and integrated with other signal transduction pathways in order to bring about cellular responses, and maintain cellular homeostasis in nonmuscle cells.

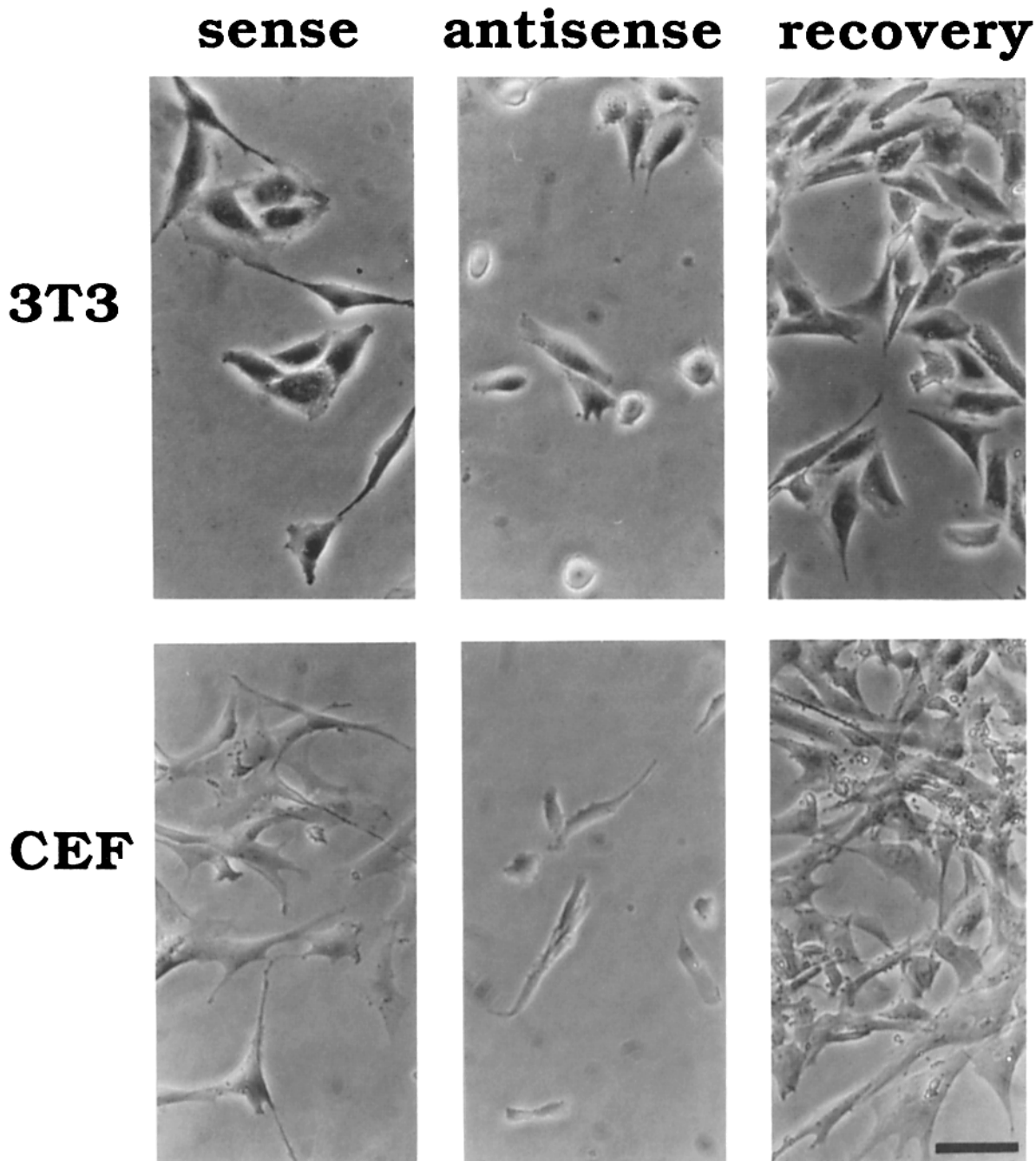
## Discussion

The results described in this report provide insight into how the primary structure of a nmMLCK is related to the transduction of a calcium signal into a biological response through protein kinase activation, indicate a role for MLCK in nonmuscle cell homeostasis, and demonstrate a structural and possible genetic relationship between nmMLCK and

smMLCK. Specifically, mapping of the CaM regulatory unit within an active MLCK, and determining how the regions are related to selective recognition and activation by CaM, provided insight into how the CaM/MLCK complex is able to transduce a calcium signal into a biological response. In particular, the results are consistent with the model of Lukas et al. (48, 50), which suggests a segmental organization of the CaM regulatory region into distinct autoinhibitory and CaM binding elements, similar to the postulated segmental organization of CaMPK-II. Further, the ability of a charge reversal mutation (rMLCK2) to suppress the functional effects of charge reversal mutations in CaM provides convincing evidence that is consistent with the hypothesis (14, 48, 50, 80) that complementary charge features of CaM and protein kinases are key to the formation and function of CaM/protein kinase signal transduction complexes. On a more general level, the functional effects of some of the mutations would not have been predicted based on precedents in structural biology and previous studies with peptide analogues and inhibitors. Relatedly, the unique nature of the amino-terminal tail segment of the nmMLCK, combined with the presence of receptor-like motifs, raised interesting possibilities about the molecular basis of subcellular targeting. Overall, the knowledge derived from the studies described here provide insight into, and a firm basis for dissecting how, an enzyme structure is related to nonmuscle cell structure and function, and how two mRNAs with extended sequence identity are apparently produced in a tissue-selective manner.

A primary goal of this study was to increase our understanding of how MLCK selectively recognizes CaM and which features of the CaM regulatory region are required for intramolecular signal transduction. Chimeric constructs, where the RS20 region (Fig. 5) of fibroblast MLCK was replaced with the corresponding regions from skeletal muscle MLCK (rMLCK6) or CaMPK-II (rMLCK7), and site-specific mutagenesis within the RS20 sequence (rMLCK2-rMLCK15) demonstrated the functional importance of the RS20 region in CaM recognition (Figs. 6, 8, and 9). Interestingly, while the RS20 region of MLCK is functionally coupled to the catalytic segment (intramolecular signal transduction between the CaM recognition and catalytic segments), the ability of the MLCKs mutated in the RS20 region to maintain substrate specificity properties demonstrates the somewhat autonomous or "cassette" nature of the segmentally organized primary structure. Similarly, the ability to extensively alter CaM recognition while maintaining autoinhibition activity demonstrated the sequential nature of the protein structure and function even within the CaM regulatory unit. In contrast, if the center of the autoinhibitory region was in the RS20 region, as has been proposed in many earlier models (discussed in the Introduction), then the mutagenesis of these residues would be expected, at least in some cases, to result in a constitutively active enzyme.

The failure to demonstrate a constitutively active enzyme for any of the mutations within the RS20 region, and the ability of charge reversal mutations in the AM13 region to produce a constitutively active enzyme, demonstrate that the AM13 region is the more likely candidate for critical residues involved in autoinhibition. Therefore, the models that are consistent with the results reported here are ones in which the CaM recognition and autoinhibitory sequences



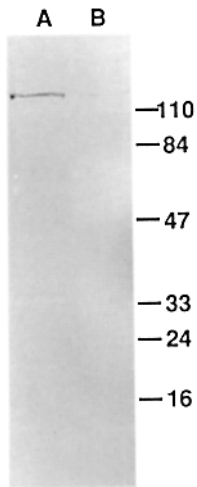
**Figure 12.** Selective and reversible effects of antisense oligodeoxynucleotides on CEF and 3T3 cells. Treatment of CEF and 3T3 cells with sense or antisense oligodeoxynucleotides (shown in Fig. 11) for 16 h resulted in a change in morphology with antisense, but not sense, oligodeoxynucleotides. Removal of antisense oligodeoxynucleotides by a change in media resulted in a restoration to normal appearance as shown by the recovery after 72 h. Cells were plated at a density of  $\sim 10^4$  cells/ml in a 24-well tissue culture plate (Falcon Labware, Oxnard, CA) and grown for 24 h in  $\alpha$ -MEM (Gibco Laboratories, Grand Island, NY) containing 10% (vol/vol) fetal calf serum (HyClone Laboratories, Logan, UT). Medium was aspirated at time zero, cells washed with serum-free  $\alpha$ -MEM, and the oligodeoxynucleotides added (30  $\mu$ M final concentration) in serum-free medium. After 2 h, serum was added to a final concentration of 2.5% (vol/vol) and cells incubated at 37°C for 16 h (41). Cells were fixed with 2.5% (vol/vol) glutaraldehyde and photographed. The effect of mismatch oligodeoxynucleotides or solvent (water) controls were indistinguishable from the sense oligodeoxynucleotides shown.

are distinct (39, 50), but intimately coupled in transducing the intramolecular signals that result in enzyme activation.

The demonstration, in this report, that the nmMLCK and smMLCK are virtually identical within the catalytic and CaM regulatory segments raises the reasonable question of why a recent report (4), dealing with a carboxy-terminal

truncation mutation of smMLCK that appears to be equivalent to rMLCK16, was not able to show CaM-independent and constitutively active enzyme activity. We do not know the basis for these apparent differences, although plausible explanations based on the results and model presented here are available. One clear difference between the studies of





**Figure 13.** Treatment of fibroblasts with MLCK antisense oligodeoxynucleotides for 16 h results in a decrease in immunoreactive MLCK levels. After treatment of CEF with sense or antisense oligodeoxynucleotides as described in Fig. 12, cells were harvested, lysed in sample buffer, and equal amounts of protein from each sample analyzed by Western blot using a polyclonal anti-MLCK antiserum. *A* is the sample from cells treated with sense oligodeoxynucleotide and *B* is from antisense oligodeoxynucleotide treatment. Molecular weight standards ( $\times 10^{-3}$ ) are indicated on the right.

Bagchi et al. (4) and ours is in the design aspects of protein engineering. The parental construct used here is rMLCK1, which is equivalent in length to skMLCK (Fig. 3), whereas the parental MK-40K construct of Bagchi et al. (4) is shorter than any naturally occurring MLCK. In addition, rMLCK1 includes a set of sequence motifs from the amino-terminal tail segment that may be important in protein targeting or folding. The MK-40K construct lacks these amino-terminal structural motifs. Relatedly, Herring et al. (33) have been able to obtain CaM-dependent activity when they express a cDNA that encodes skMLCK, although they do not obtain activity if they truncate skMLCK at a position equivalent to amino acid 1077 of nmMLCK (Fig. 2, top). From the perspective of the model presented here, which is based on studies that include the analysis of sk/nmMLCK chimeras, the results of Herring et al. (33) have alternative explanations. For example, the carboxy-terminal truncation mutation of Herring et al. (33) that lacks catalytic activity still contains 9 out of the 13 amino acids in the segment of skMLCK that is similar to AM13 (see Fig. 3). Clearly, this much of an AM13-like sequence might still function as an effective autoinhibitory region. In addition, this truncation of the skMLCK sequence also produces an MLCK that is shorter than naturally occurring MLCKs. These earlier reports also raise the possibility that the exact sequence at the carboxy-terminal end of a construct might affect protein folding. In this regard, our mutant MLCKs that were truncated within the AM13 region were less amenable to purification and characterization than those shown in Fig. 5 (unpublished observation). Overall, the model presented here for how the segmental structural organization of MLCK is related to function is not only consistent with the results presented here, it is consistent with other results that cannot be adequately explained by autoinhibition models that equate CaM binding sequences with autoinhibitory sequences.

Of the 20 mutations of rMLCK1 that were made and analyzed as part of this study, at least two provided novel results that have implications beyond the area of intracellular calcium signal transduction. These mutants are rMLCK2 and rMLCK13. The suppressor mutant rMLCK2 was designed based on previous studies which demonstrated that: (a) the RS20 region has selective, high affinity CaM binding activity (48, 50); and (b) protein electrostatics might be important

in protein recognition and calcium signal transduction by CaM:protein kinase complexes (14, 80). The production of this desired suppressor mutation at a protein/protein interaction region was based on considerations of structure, mechanism, and thermodynamics, not on random mutagenesis, screening, and phenotypic selection. Therefore, rMLCK2 has interesting features in terms of the approaches used in its design, and in terms of its potential for providing insight into how an enzyme activity can be modulated by an effector protein. However, it should be noted that the results with rMLCK2 do not require that the residues within the Arg-Arg-Lys sequence be involved in intermolecular contacts between CaM and MLCK, but they also do not preclude the possibility. Knowledge about the three-dimensional structure of a series of wild-type and mutant protein complexes is needed in order to understand how functional suppression is related to the structure of the complex.

The maintenance of function in rMLCK13, which has an inverted amino acid sequence, was both unexpected and revealing in terms of regulatory mechanism and protein recognition. We are not aware of a similar case where a functional unit in a protein has been inverted in sequence orientation with maintenance of function, although these results are reminiscent of the aspects of symmetry observed in DNA/protein recognition. Although there appears to be a lack of precedents for rMLCK13 in terms of protein/protein interaction, the peptide analogue studies of fibronectin (83) are relevant, although different in result. In this system, synthetic peptide analogues of a tetrapeptide cell adhesion region of fibronectin were made in reverse order with a maintenance of activity when the sequence was a short peptide. However, when the inverted sequence was placed into the context of a larger peptide, more like its environment when the sequence is resident in the protein, the inverted sequence was inactive. In contrast, when the CaM recognition sequence was inverted in MLCK (rMLCK13), CaM recognition and intramolecular signal transduction functions were maintained, as evidenced by the selective recognition of CaM and resultant relief of intramolecular inhibition (Fig. 7).

Overall, the ability of the CaM/MLCK complex to compensate functionally for extensive alterations in the CaM recognition region indicates that mutations within the exons encoding this region would not necessarily be lethal or always result in a major phenotypic change. In this regard, the molecular genetics and phenotypic manifestations of inherited alterations in the CaM/MLCK complex might have thematic similarities to CaM regulated ion channels, where inherited mutations of CaM must be strong enough to detect, but weak enough not to be lethal (51). The results of antisense oligodeoxynucleotide studies described in this report demonstrate that transient decreases in MLCK levels can bring about phenotypic changes that are nonlethal. However, it is possible that pathological states could result if these changes in nonmuscle cell MLCK activity are combined with changes that normally occur during development and differentiation, or if these changes in MLCK activity are combined with changes in other protein kinases or phosphatases that occur during physiological responses or cell cycle progression. Altogether, the information, tools and insights derived from this study provide a firm and more rational basis for the design and interpretation of physiological and genetic studies that seek to explore these possibilities.

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