Domain Characterization of Rabbit Skeletal Muscle Myosin Light Chain Kinase*

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Myosin light chain kinase can be divided into three distinct structural domains, an amino-terminal “tail,” of unknown function, a central catalytic core and a carboxy-terminal calmodulin-binding regulatory region. We have used a combination of deletion mutagenesis and monoclonal antibody epitope mapping to define these domains more closely. A 2.95-kilobase cDNA has been isolated that includes the entire coding sequence of rabbit skeletal muscle myosin light chain kinase (607 amino acids). This cDNA, expressed in COS cells encoded a Ca\(^{2+}\)/calmodulin-dependent myosin light chain kinase with a specific activity similar to that of the enzyme purified from rabbit skeletal muscle. Serial carboxy-terminal deletions of the regulatory and catalytic domains were constructed and expressed in COS cells. The truncated kinases had no detectable myosin light chain kinase activity. Monoclonal antibodies which inhibit the activity of the enzyme competitively with respect to myosin light chain were found to bind to residues 235–319 and 165–173, amino-terminal of the previously defined catalytic core. Thus, residues that are either involved in substrate binding or in close proximity to a light chain binding site may be located more amino-terminal than the previously defined catalytic core.

Phosphorylation of myosin regulatory light chains by Ca\(^{2+}\)/calmodulin-dependent myosin light chain kinase is thought to be responsible for the initiation of contraction in smooth muscle (Kamm and Stull, 1985) and potentiation of isometric contraction in skeletal muscle (Stull et al., 1986). Myosin light chain kinases specifically phosphorylate the regulatory light chains of myosin. Although there are several tissue and animal species specific isoforms of the enzyme, they can all be classified into one of two groups based on their antigenic and catalytic properties. The first group includes the smooth and nonmuscle isoforms and the second, the striated muscle isoforms. Myosin light chain kinases of both groups require binding of Ca\(^{2+}\)/calmodulin for maximal activity.

The best-characterized myosin light chain kinase is the skeletal muscle isoform. The entire amino acid sequence has been determined for the rabbit enzyme (603 residues, Takio et al., 1988) and predicted from the cDNA encoding the rat kinase (610 residues, Roush et al., 1988; Herring et al., 1989).

Several studies have assigned functional domains of the enzyme to specific regions within the primary sequence. Hydrodynamic and CD spectroscopic data have been used to define a shape model for the kinase (Mayr and Heilmeyer, 1983). In this model the catalytic and regulatory domains form a globular structure with a high \(\alpha\)-helix content; the remaining part of the molecule is asymmetric, contains very little \(\alpha\)-helix and is unusually rich in proline. The proposed model shows this asymmetric region protruding as a rigid rod from the globular portion. Comparison of the primary sequence of the rabbit myosin light chain kinase with that of other protein kinases suggests that the catalytic core is likely to be located between residues 302–539 (Hanks et al., 1988). Proteolysis studies have shown that limited chymotryptic digestion of the enzyme produces a 35-kDa fragment which is active but Ca\(^{2+}\)/calmodulin independent (Edelman et al., 1989). This fragment encompasses residues 256–584, thus confirming the relative position of the catalytic site. The Ca\(^{2+}\)/calmodulin-independent activity of this fragment indicates that the calmodulin-binding domain is located outside this region. A 40-kDa tryptic fragment (residues 236–594) is dependent on Ca\(^{2+}\)/calmodulin for activity. This observation together with synthetic peptide studies (Edelman et al., 1985; Blumenthal et al., 1985) has lead to the assignment of residues 577–593 as the calmodulin-binding domain of rabbit skeletal muscle myosin light chain kinase. Within this domain is an amino acid sequence with high homology to the region of the light chain substrate containing the phosphorylatable serine residue. It has been proposed that when myosin light chain kinase is inactive this homologous “autoinhibitory” region binds to the active site of the enzyme preventing it from interacting with the light chain substrate (Kennelly et al., 1987). When Ca\(^{2+}\)/calmodulin binds to the kinase a conformational change occurs in which the autoinhibitory region is removed from the active site, thus, reversing its inhibition of enzyme activity. A similar “pseudosubstrate” hypothesis has been proposed for smooth muscle myosin light chain kinase (Kemp et al., 1987; Pearson et al., 1988).

The catalytic domain of myosin light chain kinase has been further defined with monoclonal antibodies (Nunnally et al., 1987). Several antibodies have been characterized with respect to their ability to inhibit myosin light chain kinase activity and the approximate location of their epitopes. Several of these antibodies have been proposed to have epitopes located within the catalytic domain of the kinase since binding of antibody Fab fragments inhibited enzyme activity competitively with myosin light chain. We have, therefore, used these antibodies together with deletion mutagenesis studies to characterize more precisely the domains with the primary structure of the enzyme that are important for catalysis. Two interesting results were obtained. We have demonstrated that residues located between amino acids 235–319 and 165–173

* This work was supported by National Institutes of Health Grant HL06296. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank\textsuperscript{TM}/EMBL Data Bank with accession number(s) J05194.
are either part of or in close apposition to a light chain-binding site. In addition, a mutant enzyme lacking the calmodulin-binding domain and pseudosubstrate sequence but containing the catalytic domain was found to have no detectable catalytic activity and to adopt a conformation distinct from that of the native enzyme. Based upon these results, we suggest that the carboxyl terminus of the kinase is important for maintaining the native conformation of the enzyme.

**EXPERIMENTAL PROCEDURES**

**Protein Purification and Kinase Assays**—Rabbit skeletal muscle myosin light chain kinase was purified essentially as described previously (Nunnally et al., 1985) except that the Affi-Gel Blue column was replaced with a Bio-Gel A 1.5 m column (5 x 90-cm equilibrated in 10 mM MOPS, 200 mM NaCl, 0.5 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 10 mM leupeptin). Rabbit skeletal muscle myosin light chains were purified according to Blumenthal and Stull (1980). Calmodulin was purified from bovine brain (Blumenthal and Stull, 1982). The activity of myosin light chain kinase was measured by 32P incorporation into rabbit skeletal muscle regulatory (18.5 kDa) light chain as described previously (Blumenthal and Stull, 1980).

**Immunobots**—Cell extracts or purified myosin light chain kinase were electrophoresed on a 7.5% SDS-polyacrylamide gel. Following electrophoresis the proteins were transferred to nitrocellulose at 110 mA overnight in the presence of 20% methanol. The blots were reacted with antibodies (polyclonal or monoclonals 14a, 12a, 9a, and 2a, respectively) as described previously (Nunnally et al., 1987), with the exception that 0.2% nonfat dried milk was substituted for the 5% bovine serum albumin in the blocking step and antibody dilution buffers. Blots which were reacted with monoclonal antibodies 19a and 10a were blocked in 10 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween, and 1% gelatin. The antibodies were diluted in the same buffer. Following incubation the blots were washed three times for 5 min in the buffer without gelatin, then incubated in rabbit anti-mouse IgG conjugated with horseradish peroxidase. Following this incubation the wash procedure was repeated and the bound peroxidase was detected with N-nitro-blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate-tetraolide salt (Bio-Rad), according to the manufacturer's directions.

**Northern Blots**—Total cellular RNA was isolated from rapidly dissected and frozen tissue (Illaria et al., 1985). Poly (A)+ RNA was purified by oligo(dT) cellulose chromatography and Northern analysis was performed by standard procedures (Maniatis et al., 1982). Blots were probed with gel-purified cDNA that was radiolabeled with 32P by the random hexamer primer method (Feinberg and Vogelstein, 1987).

**Rabbit Skeletal Muscle cDNA Library**—An XgtlO, oligo(dT), and random hexamer primed rabbit skeletal muscle muscle cDNA library was generously provided by Dr. David Russell. This library was screened with a 32P-labeled DNA probe (Feinberg and Vogelstein, 1983) made from the EcoRI-BamHI restriction fragment (nucleotide residues 1-335) of the rat skeletal muscle myosin light chain kinase cDNA (Herring et al., 1989). Hybridization was performed at 55 °C for 16 h as described by Maniatis et al. (1982). Following hybridization the filters were washed to remove nonspecific binding, and the final wash was at 55 °C in 2.5 mM sodium phosphate, pH 7.4, 30 mM NaCl, 0.2 mM EDTA, 0.1% SDS (0.2 x SSPE) for 1 h. From this library 70 positive clones were isolated, and the largest was 1.85 kb.

**Construction of Deletion Mutants**—Purified restriction fragments of the myosin light chain kinase cDNA were subcloned into M13 Mp18, partially sequenced to verify the sequence at the 3′ end of the insert and then subcloned into pCMV 2, which was kindly provided by Dr. David Russell. This vector contains part of the promoter region from human cytomegalovirus, a synthetic polylinker, a terminal polyadenylation signal from the human growth hormone gene, and an SV 40 origin of replication (Anderson et al., 1989). Deletions 1-4 consist of restriction fragments EcoRI-AfII (1-1929), EcoRI-BamHI (1-1429), EcoRI-Stul (1-1174), and EcoRI-SacI (1-861), respectively.

**COS Cell Propagation and Transfection**—The full length cDNA or cDNA with one of the deletions described above was subcloned into the expression vector pCMV 2. The purified DNA (2 μg) was then used to transfect each 60-mm dish of COS cells (Shubita et al., 1987). Prior to transfection the cells were grown to approximately 70% confluence in Dulbecco's modified Eagle media supplemented with 10% horse fetal calf serum. After transfection the cells were incubated for 48 h before being lysed in 0.5 ml of a solution comprised of 1% Nonidet P-40 (Tergitol), 100 mM MOPS, 1.25 mM EGTA, 120 mM NaCl, 1.25 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml pepstatin at pH 7.5. The lysate was clarified by centrifugation (5 min, 14,000 × g at 5 °C), the supernatant fraction was made 10% with respect to glycerol and frozen at -70 °C in aliquots.

**RESULTS**

**Screening λgt10 Library**—Many (70) positive plaques hybridized at high stringency to the EcoRI-BamHI cDNA probe (nucleotide residues 1-335 of the rat myosin light chain kinase cDNA described in Herring et al., 1989). The phage were purified and DNA was prepared from each. The purified DNA was digested with the restriction endonuclease EcoRI and the size of the cDNA inserts determined by agarose gel electrophoresis. The largest of the inserts, a 1.85-kb cDNA, was present in several of the clones. Sequence analysis of this cDNA revealed that it encoded the carboxyl terminus of the skeletal muscle myosin light chain kinase (amino acid residues 293-607, Fig. 2; Takio et al., 1986). The sequence analysis together with the occurrence of a cDNA of 1.85 kb in several clones suggested that the 5′-EcoRI restriction site of the clone was likely to be an internal recognition site rather than one introduced by the synthetic linkers used to construct the library. A similar observation was made previously for the rat skeletal muscle myosin light chain kinase (Roush et al., 1988).

**Screening the Primer Extended cDNA Library**—In order to obtain a cDNA encoding the amino-terminal portion of the protein, an oligonucleotide was synthesized which was complimentary to residues 1,383-1,398 of the full length cDNA. This oligonucleotide was used to then construct a primer-extended cDNA library as described under “Experimental Procedures.” The resultant library consisted of approximately 1725 inserts.

1 The abbreviations used are: MOPS, 4-morpholinepropanesulfonic acid; SDS, sodium dodecyl sulfate; kb, kilobases; TWEEN, polyoxyethylene sorbitan monolaurate; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetacid.
FIG. 2. Nucleotide sequence of the cDNA encoding rabbit skeletal muscle myosin light chain kinase. The nucleotide sequence of the full length cDNA and the deduced amino acid sequence of the coding region are shown. The internal EcoRI restriction site which was used to join the two halves of the clone is located at nucleotides 1096-1101. Amino acid residues are numbered on the left of the figure and nucleotide residues on the right.

25,000 recombinant clones. We characterized 15 positive clones, including the largest 1.4-kb cDNA. This cDNA was isolated, subcloned into M13, and sequenced. The sequence analysis revealed that it encoded the amino terminus of the myosin light chain kinase (amino acid residues 1-393) together with 210 residues of 5'-untranslated sequence (Fig. 2).

Construction of a Full Length cDNA—The two halves of the cDNA were spliced together at the internal EcoRI restriction site by subcloning the gel purified fragments into M13. The resultant full length cDNA sequence is shown in Fig. 2. The mutagenic oligonucleotide described under "Experimental Procedures" was used to select for the M13 plaques which contained the two halves of the cDNA in that correct orientation. In order to facilitate subcloning of the cDNA as a single EcoRI fragment, the M13 plaques containing the myosin light chain kinase cDNA were then purified and the internal EcoRI site mutated as described under "Experimental Procedures." The mutation results in a degenerate base substitution and therefore does not effect the encoded protein sequence. This modified cDNA was used in all subsequent experiments.

Sequence Analysis of the cDNA—The cDNA sequence was aligned with that of the rat skeletal muscle myosin light chain kinase cDNA sequence (Roush et al., 1988; Herring et al., 1989). Overall, there is 70% sequence identity between these two sequences; within the coding sequences the identity is 77%. This high degree of similarity at the nucleotide level is reflected in the primary structure of the rat and rabbit kinases as discussed previously (Roush et al., 1988). Within the proposed catalytic core of the kinases (amino acid residues 302-539) the nucleotide identity is much greater. The region of greatest identity (90%) being from nucleotide 881 to nucleotide 1704 of the rabbit cDNA. This region corresponds to amino acid residues 218-495 and thus extends more amino-terminal than the previously defined catalytic core. A region between nucleotide residues 1850-2050 also displays 90% nucleotide identity between rat and rabbit cDNAs. These nucleotides encode amino acids 545-595, and thus include the proposed calmodulin-binding domain (amino acids 577-595). It is also interesting to note that nucleotides 165-219 (equivalent to -51 to +3 in the rat cDNA) are very highly conserved in both species (45 identical). This unusually high degree of identity in the 5'-untranslated sequence may indicate that this region has some important function, perhaps in regulating translation. The deduced amino acid sequence of the rabbit skeletal muscle myosin light chain kinase is identical to the reported sequence determined by amino acid analysis (Takio et al., 1986) with the exception of an additional four amino acids (A, L, G, V) present at the carboxyl-terminus of the deduced sequence.

Size and Tissue Distribution of the mRNA—The cloned cDNA hybridized to a 3.3-kb RNA (Fig. 3). The cloned cDNA is 2.95 kb, which is approximately 350 bases shorter than the estimated message size. It is, therefore, likely that the cloned cDNA is missing some 5'-untranslated sequence. Northern analysis of RNA isolated from rabbit fast and slow twitch skeletal muscle, heart, liver, and uterus demonstrated that the cDNA specifically hybridized to a 3.3-kb RNA present only in skeletal muscle (Fig. 3).

Expression of the cDNA in COS Cells—The 2.95-kb EcoRI fragment of the cDNA encoding myosin light chain kinase was subcloned into the expression vector pCMV 2 and tran...
siently expressed in COS cells. Following SDS-polyacrylamide gel electrophoresis, the expressed protein was analyzed by the immunoblot procedure with polyclonal anti-myosin light chain kinase antibodies (Nunnally et al., 1985). The antibody specifically bound to a protein of 87 kDa in extracts in which the COS cells had been transfected with cDNA in the sense orientation (Fig. 4). The recombinant protein was found to be indistinguishable in size from purified rabbit skeletal muscle myosin light chain kinase. The protein expressed in COS cells was catenatively active and Ca2+/calmodulin-dependent with a specific activity of 41 pmol of 32P incorporated/min/ 

ng enzyme (Table I). This value is very similar to that of the purified enzyme.

Construction, Expression, and Characterization of Deletion Mutants—Successive 3' deletions of the cDNA encoding myosin light chain kinase were made. Each deletion contained a common 5' terminus and utilized various restriction endonuclease sites to generate a truncated 3' terminus as described under "Experimental Procedures" (Fig. 5, Table I). This approach facilitated the construction of truncated proteins in which the calmodulin-binding domain alone or together with part or all of the catalytic domain were deleted. The truncated proteins present in the COS cell lysates were readily detected by immunoblot analysis using the polyclonal anti-skeletal muscle myosin light chain kinase antibody (Fig. 4). The precise carboxyl termini of the deletion mutants are indicated in Table I. The additional amino acids fused onto the carboxyl-terminus of each protein are encoded by vector sequences encountered before a termination codon is reached.

The truncated proteins were characterized by immunoblot analysis with several previously defined anti-myosin light chain kinase monoclonal antibodies (Nunnally et al., 1987, see Table II). Some of these antibodies (2a, 9a, and 12a) have previously been proposed to bind with the catalytic domain of myosin light chain kinase. Binding of these antibodies inhibited enzyme activity competitively with respect to light chain substrate (Nunnally et al., 1987). It is therefore possible that the epitopes for these antibodies may lie very close to a light chain-binding site within the catalytic domain of the kinase. These three antibodies together with monoclonal antibody 16a bound to all the truncated myosin light chain kinases except for the one produced by deletion 4 (Fig. 6, Table II). The remaining two antibodies analyzed (14a and 19a) bound to all the deletion mutants.

![Fig. 4. Immunoblot analysis of protein expressed from cDNAs transacted into COS cells. Wild type and truncated cDNAs were subcloned into the pCMV expression vector and transacted into COS cells as described under "Experimental Procedures." The Nonidet P-40 lysates of the transfected cells were separated on 7.5% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and reacted with polyclonal anti-skeletal muscle myosin light chain kinase as described under "Experimental Procedures." Lane 1, lysate from cells with no DNA; lane 2, lysate from cells transfected with the full length cDNA in an antisense orientation; lane 3, 30 ng of purified rabbit skeletal muscle myosin light chain kinase; lane 4, lysate from cells transfected with the full length cDNA in the sense orientation; lanes 5–8, lysates from cells transfected with cDNA deletions 1–4, respectively. The positions of protein molecular weight standards are shown on the left side of the figure.](image-url)

| Protein | COOH terminus | Specific activity*<sup>a</sup> | +Ca²⁺ | -Ca²⁺ |
|---------|---------------|-------------------------------|--------|--------|
| Purified enzyme | V(607) | 57.5 ± 12.7 | 0.06 ± 0.00 |
| Wild type | V(607) | 41.5 ± 12.0 | 0.05 ± 0.01 |

Deletion

| Deletion | Sequence | Specific activity*<sup>a</sup> | +Ca²⁺ | -Ca²⁺ |
|----------|---------|-------------------------------|--------|--------|
| 1 | K(672)-eml | None | None |
| 2 | H(405)-wyhav | None | None |
| 3 | G(319)-vasl | None | None |
| 4 | L(183)-36aa<sup>b</sup> | None | None |

* The units for specific activity are pmol of 32P incorporated/min/ 

ng enzyme.

**Fig. 5. Partial restriction map of the full length cDNA and its alignment with the schematic linear amino acid sequences of the wild type and truncated myosin light chain kinases. Restriction sites used in the construction of the truncated cDNA are shown on the nucleotide map (top). The deduced amino acid residues of the wild type and truncated proteins are aligned below the nucleotide map. Each deletion has a common, wild type amino terminus; the carboxyl terminus of each expressed kinase is indicated in the figure. The proposed catalytic (hatched area) and calmodulin-binding (shaded) domains are indicated for the wild type kinase.**

Each of the truncated proteins were analyzed for myosin light chain kinase activity. Although each lysate analyzed under standard assay conditions appeared to have some measurable kinase activity (Table I), it was only one-thousandth of the wild type enzyme. This apparent activity was also detected in COS cell lysates containing no myosin light chain kinase and occurred in the absence of added light chain.
subtracted. Thus, none of the truncated myosin light chain kinases had any measurable catalytic activity above the background of this assay system. As this was an unexpected result for the truncated protein missing only the calmodulin-binding domain and pseudosubstrate domain, the stability of this protein was further analyzed. The protein was not proteolyzed under the kinase assay conditions as determined by immunoblot using polyclonal anti-myosin light chain kinase antibodies. Properties described in Nunnally et al., 1987.

### Table II

**Characteristics of monoclonal antibodies raised to rabbit skeletal muscle myosin light chain kinase**

The binding of each monoclonal antibody to the deletion mutants is a summary of data presented in Fig. 6. ND refers to not determined. The + symbol indicates binding whereas the - symbol indicates no detectable binding.

| Property             | Monoclonal antibody |
|----------------------|---------------------|
|                      | 19a 16a 14a 12a 9a 2a |
| Inhibition of activity | - - + + + + |
| Competitive inhibition | - - + + ND + |
| Binding              |                    |
| Wild type            | + + + + + + |
| Deletion             |                    |
| 1                    | + + + + + + |
| 2                    | + + + + + + |
| 3                    | + + + + + + |
| 4                    | + + + + + + |

#### DISCUSSION

Results presented in this article document the isolation and characterization of a cDNA encoding rabbit skeletal muscle myosin light chain kinase. The cDNA is 70% identical to the rat skeletal muscle myosin light chain kinase cDNA. However, two regions of much greater identity (90%) were found. The first of these regions is located between nucleotide residues 881–1704 (Fig. 2) encoding amino acids 218–485. This region includes most of the previously defined catalytic core but also 64 more residues toward the amino terminus. The second region comprised of nucleotides 1850–2050 encodes amino acids 545–595; this region includes the proposed calmodulin-binding domain (577–595) together with an additional 32 residues toward the amino terminus. The importance of this additional highly conserved region is not known. At the amino acid level residues extending into the catalytic domain (with the exception of residues 533–543) and to the carboxyl-terminus of the protein are also equally well conserved between the two species. Secondary structure analysis by the method of Garnier et al. (1978) indicates that residues 550–582 may form an α-helix. Thus, we suggest that this region may be important in either maintaining the structure of the proposed inhibitory region and calmodulin-binding domain, or in transmitting conformational changes to the catalytic domain of the kinase. This speculation will need verification by additional experimental studies.

The predicted protein sequence encoded by the rabbit skeletal muscle myosin light chain kinase cDNA conforms the previously published amino acid sequence (Takio et al., 1986) with the exception of four addition amino acids at the carboxyl terminus of the deduced sequence. These residues are also present in the predicted rat sequence (Herrin et al., 1989).
Although it is possible that they are not present in the native protein, it is more probable that they were missed in the original protein sequence because of the small cyanogen bromide peptide in which they are contained.

Northern analysis of RNA isolated from several different rabbit tissues demonstrates that the myosin light chain kinase cDNA only hybridized to RNA isolated from skeletal muscle. Furthermore, there is a higher level of myosin light chain kinase specific RNA present in fast twitch as opposed to slow twitch skeletal muscle, a result similar to that obtained with rat tissues (Roush et al., 1988; Herring et al., 1989). This distribution reflects the relative abundance of kinase in these two tissues (Moore and Stull, 1984). Both in rat and rabbit the distinct skeletal muscle isoform of myosin light chain kinase is not detectable in other tissues, including smooth muscle.

The protein expressed from the cloned cDNA was found to have a specific kinase activity similar to the purified myosin light chain kinase. On SDS-polyacrylamide gel electrophoresis both the purified rabbit skeletal muscle myosin light chain kinase and the expressed protein have an anomalous molecular mass of 87 kDa as compared with the molecular mass of 65 kDa determined from the amino acid sequence. Thus, in terms of catalytic activity and mobility on SDS-polyacrylamide gel electrophoresis, the purified protein and the protein expressed in COS cells are indistinguishable.

Several anti-rabbit skeletal muscle myosin light chain kinase monoclonal antibodies (14a, 12a, and 2a, Table II) have been shown previously to inhibit catalytic activity competitively with respect to the light chain substrate. Two of these (12a and 2a) were found to bind to a 14-kDa V8 peptide within the catalytic domain of the kinase. Although the location of the V8 peptide is unknown, the peptide is generated from a 40-kDa tryptic peptide (residues 236-594, Takio et al., 1985; Fig. 6). Both of these antibodies bind to deletion mutants 1, 2, and 3 but not 4, thus localizing their epitopes to residues 183 and 319. These observations together with the proteolysis data discussed above, suggest that the epitopes for these two monoclonal antibodies must be located between residues 235 and 319 (Fig. 8). Monoclonal antibody 16a also binds to this same region. However, its binding does not affect the catalytic activity of the enzyme. Within this domain two distinct epitopes must therefore exist; the relatively close proximity of these epitopes would support the proposition that the epitope for antibodies 12a and 2a is directly involved in light chain binding. The location of this domain implies that the catalytic domain may extend more amino terminal than the catalytic core previously defined by homology to other protein kinases (Hanks et al., 1988). Alternatively, it remains possible that the epitopes themselves may not be light chain-binding sites but merely in close proximity to these sites and that antibody binding affects light chain binding via steric hindrance.

Because monoclonal antibodies 14a and 19a bind to all of the truncated kinases, the epitopes for antibodies must be located between residues 1 and 183. This is in agreement with earlier predictions for 19a which suggested that it bound to the amino-terminal tail portion of the enzyme. Since this antibody did not bind to either 60- or 40-kDa tryptic peptides (Nunnally et al., 1987), its epitope must therefore be located between the amino terminus and residue 150 (Fig. 8). The location of the epitope for antibody 14a, which inhibits activity competitively with respect to light chain substrate, is unexpected. Peptide-binding studies demonstrated that this antibody bound to a 60-kDa tryptic peptide (residues 150-595) but not the 40-kDa peptide (residues 236-594) produced by further proteolysis (Fig. 6). These results, together with our data, indicate that the epitope for this monoclonal antibody is located between residues 150-183. It has been shown previously that antibody 14a did not cross-react with rat myosin light chain kinase (Nunnally et al., 1987). Direct sequence comparisons between residues 150-183 of rat and rabbit enzymes revealed that only residues 165-173 demonstrated any significant heterogeneity between the two kinases. Thus, the epitope can be further defined as being most likely located between residues 165 and 173 (Fig. 8). The competitive nature of the inhibition caused by antibody binding to this region implies that it may be close to or part of the substrate-binding site. It is unlikely that this region is absolutely required for light chain binding, as the active 40-kDa tryptic peptide does not contain this epitope. These results suggest that this portion of the kinase is folded into the catalytic region, thus implying that the tail region of myosin light chain kinase may not simply be a rigid rod-like structure extending beyond the catalytic domain as previously proposed (Mayr and Heilmeyer, 1983). The data also demonstrate the potential use of deletion mutagenesis in mapping epitopes of monoclonal antibodies.

None of the truncated myosin light chain kinases expressed in COS cells exhibited significant kinase activity. This result may have been predicted for deletions 2–4 in which part or all of the catalytic domain had been removed. Proteolysis studies have suggested that removal of the inhibitory pseudosubstrate domain would result in an active enzyme which is no longer dependent on Ca"+/-calmodulin. However, the truncated kinase (deletion 1) in which only the pseudosubstrate and the calmodulin-binding domain were deleted exhibited no detectable enzyme activity. There are several possible reasons for this surprising result. For example, the truncated kinase may be unstable or have an altered conformation. The results obtained from digestion with trypsin suggest that the kinase may indeed adopt a conformation different from that of the native enzyme. Thus, the carboxy-terminal (573–607) of the protein may be important for conformational stability and thereby indirectly be important for catalytic activity. Consistent with this proposition, it has been shown that the 35-kDa chymotryptic fragment (256–584) is unstable at 30 °C, whereas the 40-kDa tryptic fragment (236–594) is stable at 30 °C in the absence of calmodulin. These data suggest that the calmodulin domain may be responsible for stabilizing the conformation of the enzyme. Alternatively, if the pseudosub-

Fig. 8. Summary of epitope maps and domain organization of rabbit skeletal muscle myosin light chain kinase. This schematic diagram depicts the location of the functional domains of skeletal muscle myosin light chain kinase. The calmodulin-binding domain (Edelman et al., 1985; Blumenthal et al., 1985) is shaded, the catalytic core defined by homology with other protein kinases (Hanks et al., 1988) is hatched, whereas the catalytic domain extended to include a putative light chain binding site defined by our studies is cross-hatched. The location of the epitopes of each monoclonal antibody are indicated. The stippled area represents potential sites of high antigenicity as predicted from the hydrophilic profile of the amino acid sequence.
strate does not function simply as a substrate analogue inhibitor but also functions to transmit a conformational change during the activation process, then removal of this sequence would be predicted to result in an inactive enzyme. More detailed studies will be required to elucidate the roles of the carboxyl-terminal amino acids in the regulation and activity of myosin light chain kinase.

In summary, we have shown that a cloned cDNA encodes a full length rabbit skeletal muscle myosin light chain kinase. Expression of the cDNA in COS cells results in a myosin light chain kinase that has catalytic and immunological properties identical to the native enzyme purified from skeletal muscle. The monoclonal antibody binding data suggest that residues 235–319 and 165–173 may form part of, or be closely apposed to a light chain-binding site. We also suggest that residues 573–607 may be important for conformational stability of the kinase as well as activation of the enzyme by Ca²⁺/calmodulin.

Acknowledgments—We wish to thank Dr. David Russell for his help and advice as well as generously providing us with the rabbit skeletal muscle cDNA library and the pCMV 2 expression vector, Dr. Peter Kennelly and Dr. Edwin Krebs for generously sharing research results on inactivation of myosin light chain kinase by Ca²⁺/calmodulin in the absence of substrates, Suzy Griffin for her expert technical assistance, and Phyllis Foley for her help in preparation of this manuscript.

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