Molecular Characterization of a Mammalian Smooth Muscle
Myosin Light Chain Kinase*

Patricia J. Gallagher‡, B. Paul Herring, Suzy A. Griffin, and James T. Stull
Department of Physiology, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75235-9040

Abstract
A 5.6-kilobase cDNA clone has been isolated which includes the entire coding region for the myosin light chain kinase from rabbit uterine tissue. This cDNA, expressed in COS cells, encodes a Ca\(^{2+}\)/calmodulin-dependent protein kinase with catalytic properties similar to other purified smooth muscle myosin light chain kinases. A module (TLKPVGNIKPAE), repeated sequentially 15 times, has been identified near the N terminus of this smooth muscle kinase. It is not present in chicken gizzard or rabbit skeletal muscle myosin light chain kinases. This repeat module and a subrepeat (KP A/V) are similar in amino acid content to repeated motifs present in other proteins, some of which have been shown to associate with chromatin structures. Immunoblot analysis after sodium dodecyl sulfate-polyacrylamide gel electrophoresis, used to compare myosin light chain kinase present in rabbit, bovine, and chicken smooth and nonmuscle tissues, showed that within each species both tissue types have myosin light chain kinases with indistinguishable molecular masses. These data suggest that myosin light chain kinases present in smooth and nonmuscle tissues are the same protein.

In smooth muscle phosphorylation of the regulatory light chain of myosin by the Ca\(^{2+}\)/calmodulin-dependent MLCK\(^1\) is a well characterized event in the initiation of contraction (Kamm and Stull, 1985; Stull et al., 1989; Hai and Murphy, 1989). This event is regulated by the concentration of cytosolic Ca\(^{2+}\) which increases upon agonist stimulation of smooth muscle cells. Ca\(^{2+}\) binds to calmodulin and the Ca\(^{2+}\)-calmodulin complex then binds to MLCK and activates the enzyme. Activation of MLCK results in phosphorylation of the 20-kDa regulatory light chain subunit on myosin and stimulation of the actin-activated myosin MgATPase activity. These events lead to force generation or shortening of the muscle.

Although the role of MLCK in nonmuscle cells is not well defined, a variety of morphological changes such as cellular motility and organelle movement occurs concurrently with the increases in cytoplasmic Ca\(^{2+}\) levels, light chain phosphorylation and activation of MLCK (Yearna et al., 1979; Bissonnette et al., 1989; Burnham et al., 1988; Saitoh et al., 1986; Cande and Ezzell, 1986; Hassell et al., 1986; Masuda et al., 1984; Wagner et al., 1985; Wysolmerski

---

*This work was supported in part by Biomedical Research Support Grant BRSG 2 S07 RR 07175 (to P. J. G.) and by National Institutes of Health Grant HL26043 (to J. T. S.). 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.

‡ To whom correspondence and reprint requests should be addressed: Dept. of Physiology, University of Texas Southwestern Medical Center at Dallas, 5323 Harry Hines Blvd., Dallas, TX 75235-9040. Tel.: 214-688-868; FAX: 214-688-8685.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) M76233 and M76369.

\(^1\)The abbreviations used in this article are: MLCK, myosin light chain kinase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; bp, base pair; kb, kilobase; APMSF, amidino-phenylmethylsulfonyl fluoride; PMSF, phenylmethylsulfonyl fluoride; TLCK, N\(^\alpha\)-p-tosyl-L-lysine chloromethyl ketone; TPCK, tosyl-phenylalanyl chloromethyl ketone; MOPS, 4-morpholinepropanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid. Amino acid residues are abbreviated using the single-letter code.
and Lagunoff, 1990). Intracellular localization studies performed in mammalian fibroblast cells have colocalized MLCK to the spindle apparatus and midbody of mitotic cells, and to stress fibers and the nucleolus of interphase cells (Guerriero et al., 1981). These observations have led to the suggestion that the phosphorylation of myosin light chain by MLCK in nonmuscle cells might have a role in cell division and cell motility.

MLCKs have been characterized from a variety of smooth muscle and nonmuscle tissues (Hassell et al., 1986; Wagner et al., 1985; de Lanerolle et al., 1987; Bissonnette et al., 1989; Shoemaker et al., 1990; Adelstein and Klee, 1981; Guerriero et al., 1981). Of these tissue types, MLCK present in chicken gizzard smooth muscle is the best characterized enzyme. Immunological studies utilizing polyclonal antibodies directed against the chicken smooth muscle MLCK have shown that these antibodies recognize both smooth and nonmuscle MLCKs from a variety of animal species (Bissonnette et al., 1989; de Lanerolle et al., 1987). In general, MLCKs from mammalian smooth muscle and nonmuscle tissues have larger molecular masses (138–155 kDa) compared to those present in avian tissues (130 kDa).

cDNA clones encoding a chicken gizzard smooth muscle (Guerriero et al., 1986; Olson et al., 1990) and a chicken embryo fibroblast (Shoemaker et al., 1990) MLCKs have been identified; comparison of their nucleotide and deduced amino acid sequences suggests that the mRNAs encoding these proteins are derived from the same gene. The only significantly different property between the deduced sequences of these two kinases is the presence of an additional 286 amino acids at the N terminus of the chicken embryo fibroblast MLCK providing a molecular mass that is about 32 kDa larger than the avian smooth muscle MLCK. The domain organization of the chicken gizzard MLCK includes a central catalytic core followed by an autoregulatory and calmodulin-binding domain (Guerriero et al., 1986; Olson et al., 1990). It has been proposed that approximately 155 residues located C-terminal to the calmodulin-binding domain in MLCK are expressed as an independent protein. This protein has been purified from turkey gizzard and named telokin (Ito et al., 1989). The function of the C terminus of MLCK and its affiliated protein, telokin, are at present unknown.

One objective of this study was to identify primary structural differences and similarities existing between avian and mammalian smooth muscle MLCKs. All previous molecular characterizations have used a cDNA encoding the avian smooth muscle MLCK. A direct comparison of the primary structures of a MLCK from a mammalian species to the avian MLCK would assist in the identification of conserved and nonconserved regions which might be important functional or structural domains. A second objective of this study was to ask what, if any, are the differences between the nonmuscle and the smooth muscle MLCKs.

**Experimental Procedures**

**Northern Blot Analysis**

RNA was isolated from rapidly dissected, quick-frozen tissues, and Northern blots were prepared and analyzed (Ilaria et al., 1985). The blots were probed with 32P-labeled, random-primed cDNA fragments (Feinberg and Vogelstein, 1983). Hybridization, washing and autoradiography were as described (Sambrook et al., 1989). Hybridization was performed at 68 °C and final high stringency wash conditions were in 2.5 m NaH2PO4, 0.2 m NaCl, 0.2 m EDTA (0.2 × SSPE) and 0.1% SDS at 68 °C. Low stringency hybridization and wash conditions were performed at 55 °C in 2 × SSPE and 1% SDS. Poly(A+) RNA was prepared by affinity chromatography on oligo dT cellulose (Sambrook et al., 1989).
Preparation and Screening of Rabbit Uterine cDNA Libraries

RNA was isolated from uteri from retired New Zealand White female breeding rabbits. Poly(A\(^+\)) RNA was prepared by affinity chromatography (Sambrook et al., 1989). This poly(A\(^+\)) RNA was used to prepare an oligo(dT)-primed cDNA library in \(\lambda\)gt11 by the method of Gubler and Hoffman (1983). Recombinant \(\lambda\)-gt11 plaques were screened with a polyclonal antibody raised to bovine tracheal MLCK (Kamm et al., 1987), which had been preadsorbed with *Escherichia coli* extract (Sambrook et al., 1989).

Two additional smooth muscle MLCK specific, oligonucleotide-primed libraries were prepared with minor modifications as described by Herring et al. (1990). The oligonucleotide primers for these libraries corresponded to bp 2945–2962 (SMPE-I, AGGATGTGCAGATGACG) and bp 1385–1405 (SMPE-II, TTCCCGTTCAGTCCAGGTG). SMPE-I is located 333 bp from the 5' end of the lambda gt11 partial cDNA clone (2995 bp, Fig. 1). The SMPE-I specifically primed library was screened with a 264-bp fragment corresponding to bp 2613–2877 of the rabbit uterine smooth muscle MLCK. The SMPE-II specifically primed library was screened with a 118-bp probe corresponding to bp 1194–1312 of the smooth muscle MLCK. \(\lambda\)-DNA was prepared from positive plaques, digested with EcoRI, sized, subcloned, and sequenced. Probes used to screen these specifically primed cDNA libraries were \(^{32}\)P-labeled, random-primed cDNA fragments (Feinberg and Vogelstein, 1983).

Assembly of the Rabbit Uterine Smooth Muscle MLCK cDNA

The full-length cDNA molecule was assembled as follows. The 537-bp SalI-EcoRI fragment (bp 2340–2877) isolated from the SMPE-I-primed library and was ligated to the 1120-bp EcoRI fragment (bp 2878–3998) isolated from the \(\lambda\)-gt11 oligo(dT)-primed library to form a C-terminal fragment in pGEM 9Z (Figs. 1, B and C). The N-terminal fragment was assembled by ligation of a 1248-bp EcoRI-AlwNI fragment (bp 1–1248) isolated from the SMPE-II-primed library to a 1589-bp AlwNI-EcoRI fragment (bp 1248–2877) isolated from the SMPE-I-primed library into pGEM 7Z (Fig. 1B). The N-terminal fragment was digested with KpnI and XmnI (bp 2481). The C-terminal fragment was digested with XmnI (bp 2481) and XbaI. The 5' KpnI and 3' XbaI sites are derived from pGEM 7Z and pGEM 9Z, respectively. Ligation of these two fragments at the XmnI site generated a 3998-bp cDNA which was inserted in the KpnI and XbaI sites of pCMV 5. pGEM vectors are commercially available from Promega (Madison, WI). pCMV 5 DNA is a derivative of pCMV 2 (Andersson et al., 1989).

Isolation of the Rabbit Smooth Muscle MLCK Gene

A rabbit genomic DNA library (Clontec, Palo Alto, CA) was screened with a portion of the rabbit uterine smooth muscle MLCK cDNA (bp 649–1405). \(\lambda\)-DNA was prepared from positive plaques, and the inserts were excised by digestion with SalI and subcloned into pGEM. The genomic DNA inserts were mapped by digestion with restriction endonucleases and subcloned for DNA sequencing using synthetic oligonucleotides. The portion of the rabbit smooth muscle MLCK gene which was sequenced for this study begins 869 bp from the 5' end of the cDNA and overlaps 377 bp of the cDNA. The sequence information from this portion of the rabbit gene for MLCK was used to predict a transcriptional start site and to aid in the resolution of the 5' translational start site for the cDNA clone.

Primer Extension

Primer extension was performed essentially as described (Sambrook et al., 1989) using an oligonucleotide corresponding to bp 893–928 (Fig. 3). The oligonucleotides were labeled with T4 polynucleotide kinase and \([^{32}\)P]ATP, purified and hybridized to 50 \(\mu\)g of total RNA from rabbit uterus. The primer was then extended in a first strand cDNA reaction by reverse

*J Biol Chem.* Author manuscript; available in PMC 2010 March 11.
transcriptase. The length of the resulting end-labeled cDNA was estimated by electrophoresis through a denaturing polyacrylamide gel and comparison to radioactive molecular weight standards.

**Preparation of λ-DNA**

Positive plaques which were initially identified during screening of λ libraries were replated and rescreened until a single positive recombinant plaque was obtained. λ-DNA was then isolated from these plaques by the use of LambdaSorb phage adsorbent as described by the manufacturer (Promega). This method yielded DNA which was easily digested with restriction endonucleases. Recombinant λ-cDNA clones were end-labeled with \([32P]dATP\) prior to agarose gel electrophoresis to identify all EcoRI fragments present in the cDNA insert.

**DNA Sequencing**

Fragments of the cDNA or genomic clone were subcloned into pGEM and M13 vectors for double-stranded (Mierendorf and Pfeffer, 1987) and single-stranded sequencing by the dideoxy method (Sanger et al., 1977) using Sequenase version 2.0 and pyrophosphatase (U. S. Biochemical). Convenient restriction sites as well as synthetic oligonucleotide sequencing primers were used to resolve the DNA sequence. All DNA sequences were determined more than once, either on separate strands or from different endpoints on the same strand. Approximately 90% of the sequence was obtained from both strands. Much of the DNA sequence was read from 8% acrylamide, 40% formamide, 7 M urea gels (U. S. Biochemical Corp., 1990).

**Data Base Searches and Sequence Analysis**

Overlapping sequence data were assembled with the Assemgel program, alignments were produced with Clustal program, and secondary structure predictions were obtained with Flexpro and AAScale programs of Intelligenetics PC GENE. Alignments were refined using personal judgment. Data base searches used The University of Wisconsin Genetics Computer Group programs (Devereux et al., 1984). Wordsearch and Segments used the Wilbur and Lipman search for similarity (Wilbur and Lipman, 1983; Lipman and Pearson, 1985). The data bases GenBank™ (Release 66), EMBL (Release 26), and Swiss Prot (Release 17) were searched.

**Expression of Rabbit Smooth Muscle MLCK in COS Cells**

A portion of the cDNA (nucleotide 1–3998) which included all of the 5′ noncoding region and 222 bp of 3′ noncoding region was subcloned into the KpnI and XbaI sites of pCMV 5 to generate a vector (pCMV5-SMMLCK) for the expression of the rabbit uterine smooth muscle MLCK in COS cells. Purified DNA (2 μg) was then used to transfect a 60-mm dish which had been seeded with 6 × 10⁵ Cos cells using a DEAE-dextran transfection procedure (Sambrook et al., 1989). Following transfection the cells were incubated for 48 h before being lysed in 0.4 ml lysis buffer (10% glycerol, 1% NP-40, 10 mM MOPS, 1.25 mM EGTA, 10 mM dithiothreitol, 50 mM MgCl₂, 120 μg/ml TPCK and TLCK, 100 μg/ml PMSF, 10 μg/ml each of pepstatin, APMSF, leupeptin, and 4 μg/ml aprotinin). The detergent lysate was clarified by centrifugation (2 min, 15,000 × g at 4 °C), and the supernatant fraction was aliquoted, rapidly frozen in liquid nitrogen and maintained at −70 °C until electrophoresis. The concentration of the expressed recombinant rabbit uterine smooth muscle MLCK protein in COS cell extracts was determined by quantitative scanning densitometry of Western photoblots (described below) with purified bovine tracheal MLCK as a standard. Quantitation of recombinant proteins was performed using a monoclonal antibody directed against bovine tracheal MLCK (Kamm et al., 1987).
Preparation of Tissue and Cultured Cell Extracts

All tissues used in this study except rabbit tracheal tissue were dissected to remove adventitia and immediately frozen in liquid nitrogen. Rabbit trachea, including cartilage, were frozen and homogenized without dissection. Frozen tissues (10 mg) were homogenized in 400 μl of homogenization buffer (20% sucrose, 125 mm Tris-HCl, pH 6.8, 10% SDS, 120 μg/mL TPCK and TLCK, 100 μg/ml PMSF, 10 μg/ml each of pepstatin, APMSF, and leupeptin, and 4 μg/ml aprotinin), centrifuged (2 min, 15,000 × g, 4 °C) and the supernatant fraction was aliquoted and rapidly frozen in liquid nitrogen. The fractions were maintained at −70 °C until electrophoresis. Samples were prepared for electrophoresis by the addition of dithiothreitol to a final concentration of 50 mM, bromphenol blue to a final concentration of 0.01% and then boiled for 2 min. Protein determinations were performed on samples which did not contain dithiothreitol with the bicinchoninic acid protein assay method (BCA Protein Assay; Pierce, Rockford, IL) according to the manufacturer’s directions. Chicken embryo fibroblasts (CRL 1590), obtained from the American Type Culture Collection (Rockville, MD) were cultivated in 90% Dulbecco’s modified Eagle’s medium, 10% fetal calf serum, 0.01% tryptose phosphate broth. Cells were lysed by the addition of 0.4 ml lysis buffer, as described in the previous section.

Western Immunoblots

Proteins in tissue and cell extracts were separated by electrophoresis through discontinuous SDS-polyacrylamide gels (7% acrylamide). To detect subtle differences in the masses of immunoreactive proteins it was necessary to allow electrophoresis to continue for 30 min after the bromophenol blue dye front had reached the end of the gel. Following electrophoresis, the proteins were transferred to nitrocellulose at 90 mA for 14–16 h in the presence of 20% methanol. Blots were treated with 2% gelatin in TBST (10 mM Tris, 7.5; 150 mM NaCl; 0.05% Tween-20) and reacted either with a polyclonal or a monoclonal antibody (Kamm et al., 1987) raised against bovine tracheal MLCK. Either antibody used in this study was diluted 1:5,000 in 2% gelatin in TBST. After several washes in TBST (30 min total) the blots were reacted with goat anti-rabbit or goat anti-mouse IgG conjugated with horseradish peroxidase second step antibody diluted 1:10,000 in 2% gelatin in TBST. Following extensive washing (four changes of buffer for a total of 60 min) the immunoreactive proteins were visualized on film with the enhanced chemiluminescent detection system (ECL Western Blotting detection system, Amersham). ECL detection was performed according to the manufacturer’s directions. The quantity of expressed MLCK present in cell lysates was determined by densitometric scanning of immunoblots on which various amounts of cell extracts were compared with known amounts of purified bovine tracheal MLCK. Quantitation was performed with an immunoblot exposure where absorbance was proportional to the amount of kinase applied to the gel.

Protein Purification and Myosin Light Chain Phosphorylation Assays

MLCK from bovine trachea (Stull et al., 1990) and chicken gizzard (Adelstein and Klee, 1981) were purified as described previously. Chicken gizzard light chains were purified according to Hathaway and Haeberle (1983). Calmodulin was purified from bovine testes as described by Blumenthal and Stull (1982). The MLCK activity in extracts of COS cells which had been transfected with pCMV5-SMLCK was measured by 32P incorporation into the regulatory light chain of myosin purified from chicken gizzards. The reaction was performed in the presence of 50 mM MOPS, 10 mM magnesium acetate, 0.3 mM calcium chloride, 1 μM calmodulin, 2 mM dithiothreitol, 1 mM [γ-32P]ATP, and indicated concentrations of light chain (Blumenthal and Stull, 1980; Miller et al., 1983). The light chain concentration was calculated from the maximum 32P incorporation into gizzard light chain after prolonged incubation with [γ-32P]ATP and bovine tracheal MLCK. Background rates of incorporation of 32P into light chain determined in the presence of Ca2+/calmodulin and light chains for mock (pCMV5)
transfected COS cells were determined in a parallel transfection and subtracted from rates of incorporation of cells transfected with pCMV5-SMMLCK. $K_m$ and $V_{max}$ values were determined from Lineweaver-Burke double reciprocal plots. Cell extracts from mock (pCMV5 DNA) or pCMV5-SMMLCK DNA transfected COS cells were routinely assayed for kinase activity at dilutions ranging from 1:25–1:50. Mock transfected (pCMV5) COS cell extracts had no detectable kinase activity in control assays at low dilutions (1:5 and 1:10) in the presence of EGTA and was approximately 7% of the total kinase activity detected in the presence of Ca$^{2+}$, calmodulin, and light chain.

Protein Sequencing

Although the N terminus of purified bovine tracheal MLCK was found to be blocked, sequence data were obtained from fractionated peptides. Purified bovine tracheal MLCK was treated with either staphylococcal V8 protease (Boehringer Mannheim) or 70% formic acid (Landon, 1977), and the resultant fragments were separated by electrophoresis on SDS-PAGE (10% acrylamide). Following electrophoresis the digested protein was transferred to Immobilon™ membrane (Millipore Corp., Bedford, MA), stained with Coomassie Blue, and the fragments were cut from the membrane and sequenced (Matsudaira, 1987). Automated Edman degradation was performed with an Applied Biosystem Inc. (Foster City, CA) model 470A Sequencer.

Results

Isolation and Characterization of a cDNA Encoding Mammalian Smooth Muscle MLCK

A 5608-bp cDNA encoding a mammalian smooth muscle MLCK from rabbit uterus has been isolated and characterized. This cDNA was assembled from three overlapping fragments as shown in Fig. 1. Initially, a 2997-bp cDNA corresponding to the 3′ portion of the rabbit uterine smooth muscle MLCK was isolated by screening a λ-gt11 expression library with a polyclonal antibody directed against the bovine tracheal MLCK. Subsequent screening of two MLCK specific λ-gt10 libraries which were generated by priming the cDNA synthesis with specific oligonucleotides was required to complete the 5608-bp cDNA.

The longest open reading frame predicts this cDNA encodes a protein of 1147 residues, starting with a methionine at bp 306 and terminating at bp 3746. A polyadenylation signal (AATAAA) at bp 5571 precedes the poly(A) tail (Proudfoot, 1991). The DNA sequence and the deduced amino acid sequence of the rabbit uterine smooth muscle MLCK are presented in Fig. 2. The enzyme has a predicted molecular mass of 125,719 Da. The sequence of peptides obtained from purified bovine tracheal MLCK corresponds to several regions in the amino acid sequence predicted from the rabbit uterine smooth muscle MLCK cDNA (Fig. 2).

Analysis of the DNA sequence from a portion of the rabbit smooth muscle MLCK gene did not reveal the presence of any additional in-frame methionine residues within the potential open reading frame other than the proposed start site. However, within approximately 182 bp from the end of this cDNA are located several in-frame stop codons (Fig. 3). Examination of the nucleotide sequence of this region of the genomic DNA clone suggests that a putative promoter region is located between bp 661–747. Results from a primer extension experiment (data not shown) utilizing a primer located between bp 893–928 indicates that the mRNA for the rabbit uterine smooth muscle MLCK extends approximately 150 bp beyond the 5′ end of the cDNA (bp 869 of genomic sequence, Fig 3). These data suggest that one of the CA sequences located between bp 697–698 or bp 721–722, may be the mRNA cap site for the smooth muscle MLCK. Both of these CA sequences are within 30–35 bp of putative TATA sequences (Maniatis et al., 1987; Wasylyk, 1988). The size of this cDNA (5608 bp) is close to the estimated size of the mRNA detected in total and poly(A+) rabbit uterine RNA on Northern
blots (Fig. 4, A and B). Taken together, the primer extension data, the size of mRNA on Northern blots, and the length of the cDNA all suggest that the cDNA for MLCK is near full-length and lacks approximately 150 bp from the 5’ noncoding region.

**Northern Blot Analysis**

Portions of the cloned DNA were used as probes for RNA isolated from rabbit uterus and smooth muscle tissues from other species on Northern blots. A probe derived from the N terminus (bp 574–1405) of the cDNA encompassing the highly repeated region, hybridized to a single mRNA of 5.8 kb in rabbit uterine RNA. The N-terminal probe was seen to cross-react to a 5.4-kb mRNA in rat uterus, and two mRNAs of 8.4 and 5.8 kb in bovine tracheal smooth muscle tissues (Fig. 4, lanes 1, 2, 3). The N-terminal probe did not cross-hybridize to RNA prepared from chicken gizzard or rabbit skeletal muscle mRNA (not shown), even under low stringency hybridization and washing conditions. The 8.4-kb mRNA detected in bovine tracheal tissue has also been detected by probes corresponding to the catalytic region and the C terminus of MLCK. It is possible that this mRNA encodes a larger form of MLCK which is present in bovine tracheal tissue, although we have not detected a larger mass protein in this tissue by Western blotting. In contrast, a probe corresponding to a portion of the C-terminal coding region and 3’ noncoding region (bp 3350–3998) of the rabbit smooth muscle MLCK hybridized under stringent conditions to a 5.8-, 2.6- and 2.4-kb mRNA species (Fig. 4, lane 4). The 5.8-kb mRNA encodes the smooth muscle MLCK and the smaller mRNA encodes telokin. Telokin is expressed in mammalian (Gallagher and Herring, 1991) as well as in avian smooth muscle tissues (Ito et al., 1989).

**Expression of the Recombinant Rabbit Uterine Smooth Muscle MLCK**

A 4.0-kb KpnI-XbaI fragment (Fig. 1) was subcloned into the expression vector pCMV 5 (pCMV5-SMMLCK) and transfected into COS cells. Following SDS-PAGE (7% acrylamide) the expressed recombinant protein was analyzed by an immunoblot procedure with polyclonal antibodies directed against the bovine smooth muscle MLCK (Kamm et al., 1987). The antibodies specifically bound to a single protein of 152 kDa present in transfected COS cell extracts; this protein was indistinguishable in size to an immunoreactive protein present in rabbit uterine tissue extracts (Fig. 5A). The rabbit uterine smooth muscle MLCK was slightly smaller than the bovine tracheal MLCK (155 kDa). Measurements of the catalytic activity of the recombinant enzyme in COS cell extracts showed that the expressed protein was catalytically active and Ca\(^{2+}\)/calmodulin-dependent with a specific activity of 34 pmol of \(^{32}\)P incorporated/min · ng of enzyme (Table I) and a \(K_m\) for chicken gizzard light chain of 9 \(\mu\)M. These values are very similar to previously reported values for the chicken gizzard and bovine tracheal smooth muscle enzymes (Taylor and Stull, 1988; Walsh et al., 1982; Adelstein and Klee, 1981).

**Immunoblot Analysis of Smooth and Nonmuscle Tissues**

Extracts of smooth and nonmuscle tissues and a chicken embryo fibroblast cell line were analyzed by an immunoblot procedure with polyclonal antibodies directed against the bovine smooth muscle MLCK (Kamm et al., 1987). Although the masses of MLCK vary slightly, it is evident that the masses of MLCKs from rabbit and bovine tissues are greater than those detected in avian tissues and that within a species the MLCKs present in the smooth and nonmuscle tissues have an indistinguishable molecular mass (Fig. 5B). For example, the relative molecular mass of MLCK detected in rabbit uterus, trachea, aorta, and ileum smooth muscle tissues is the same (152 kDa) as that detected in kidney and adrenal nonmuscle tissues. Not shown in this figure are immunoblots detecting MLCK in rat uterine tissue. A summary of molecular masses as determined by immunoblotting after SDS-PAGE for these species is shown in Table II.
Examinations of nucleic acid and protein data bases reveal that the rabbit uterine smooth muscle MLCK is highly related to the chicken gizzard MLCK (Olson et al., 1990) and chicken embryo fibroblast MLCK (Shoemaker et al., 1990). A comparison of the nucleotide sequences of these three cDNAs shows an overall similarity of approximately 65%. In this analysis it was discovered that within the 5′ noncoding region (nucleotide 136–305, Fig. 2) of the rabbit uterine smooth muscle MLCK cDNA a high degree of similarity (83%) is maintained to the analogous sequences in the 5′-noncoding region of the chicken gizzard (nucleotides 134–302) and within the N-terminal coding region of the chicken embryo fibroblast (nucleotides 1351–1520) MLCK. Also identified were two unreported (Shoemaker et al., 1990) sequence discrepancies which occur between the two avian cDNAs which are nearly identical within this region. Between nucleotides 127–130 and 128–129 of the chicken gizzard cDNA are two additional nucleotides and a single nucleotide deletion. These sequence differences account for the presence of several in-frame stop codons in the 5′-noncoding region of the chicken gizzard MLCK, while an open reading frame is maintained in the chicken embryo fibroblast sequence. Resolution of the nucleotide sequence discrepancies between the avian cDNAs will be required to determine if these differences are due to sequencing or cloning artifacts. The additional 286-amino acid open reading frame, which is maintained in the chicken embryo fibroblast sequence, makes it unlikely that a sequence error has been made.

A comparison of the predicted amino acid sequences of rabbit smooth, chicken smooth and chicken nonmuscle MLCK was made to further identify regions of conserved residues and functional subdomains of these proteins. Fig. 6 displays an alignment of both avian and mammalian smooth and nonmuscle MLCKs. Residues 1–286 of the chicken embryo fibroblast MLCK were not included in this alignment because no analogous region exists in either the chicken or rabbit smooth muscle MLCKs. A catalytic core encompassing the eleven subdomains common to the superfamily of protein kinases (Hanks et al., 1988) is located between residues 703–939 (Fig. 6). A regulatory region including residues which have been implicated in pseudosubstrate regulation of the kinase and calmodulin binding (residues 964–990) is located C-terminal of the catalytic core (Guerriero et al., 1986). Two general regions of conservation are found between residues 1–75 in the N terminus and residues 320–1147 in the C terminus.

It appears that residues 76–141 and 362–426 of the chicken smooth and nonmuscle MLCKs, respectively, are absent in the rabbit uterine smooth muscle MLCK. Similarly, residues 76–320 of the rabbit uterine MLCK do not appear in the avian MLCKs. The amino acid content of both of these regions is enriched in K and P. It is possible that these two blocks of residues may represent analogous exons which have diverged in the mammalian and avian forms of the smooth and nonmuscle MLCKs. Based upon the alignment shown in Fig. 6 the rabbit smooth muscle MLCK is 83% identical and 97% similar (excluding residues 76–320 of the rabbit uterus, residues 76–141 of the chicken gizzard MLCK, and residues 1–286 and 362–427 of the chicken fibroblast MLCKs) to the previously described chicken gizzard MLCK (Olson et al., 1990) and chicken embryo fibroblast nonmuscle MLCK (Shoemaker et al., 1990).

Several repeated motifs, called motif I and motif II, have been described (Benian et al., 1989; Labiet et al., 1990; Olson et al., 1990) which are present in the noncatalytic domains of the smooth and nonmuscle MLCKs as well as many other proteins (Benian et al., 1989; Labiet et al., 1990). It has been proposed that these motifs have a structural role involved in maintaining myosin based filaments. Motifs I and II are also conserved in the rabbit uterine smooth muscle MLCK molecule as well as motif III which has recently been identified (Shoemaker et al., 1990).
In contrast, a comparison (data not shown) of the rabbit uterine smooth muscle and rabbit skeletal muscle (Herring et al., 1990) MLCKs revealed 58% similarity in the catalytic cores and 53% similarity in the calmodulin-binding region. There was no significant similarity between the N terminus (residues 1–702 of the rabbit uterus MLCK) of these two protein kinases. A repeated sequence module similar to that present near the N terminus of the rabbit uterine MLCK does not appear in the rabbit skeletal MLCK.

Characterization of an Unusual Repeated Motif in the N Terminus of the Rabbit Uterine Smooth Muscle MLCK

Fifteen tandem amino acid repeats (residues 112–288; Fig. 7) are located between two conserved regions near the N terminus of the rabbit smooth muscle MLCK. The repeat unit in this motif is 12 residues in length except for one unit. However, a three residue subrepeat having the consensus sequence K P (A/V) can be identified which occurs twice within each 12-residue repeat unit. Secondary structure programs predict this region is hydrophilic with a mixed α-helical, extended coil structure (Karplus and Schulz, 1985; Kyte and Doolittle, 1982; Chou and Fasman, 1978).

Searches of a protein data base with the single repeat module (12 residues) and with the 3-residue subrepeat have identified several proteins with repeat motifs having similar amino acid sequence. These proteins have multiple, tandem, or segmented repeating units which are rich in K, S (or T) A, and P residues. Examples of proteins having similar repeated motifs are shown in Table III.

Discussion

This report details the first characterization of the primary structure of a mammalian smooth muscle MLCK. The length of the cDNA (5608 bp) is similar to that of the 5.8-kb mRNA detected by Northern blot analysis. Within the cDNA sequence a single open reading frame of 1147 residues can be identified which encodes a protein having a calculated mass of approximately 126 kDa. Transfected COS cell extracts expressing the recombinant rabbit uterine smooth muscle MLCK show a single immunoreactive protein of 152 kDa which comigrates with rabbit uterine tissue MLCK. This discrepancy in masses is probably due to aberrant migration upon electrophoresis in the presence of SDS. A similar difference in calculated mass (107 kDa) and apparent molecular mass (130 kDa) is observed for the chicken gizzard MLCK (Olson et al., 1990). Inspection of genomic DNA sequence from the 5' end of the rabbit smooth muscle MLCK gene has identified several in-frame termination codons and a putative transcriptional start site which is located approximately 150 bp from the 5' end of the cDNA. These data are all in agreement with the proposal that this 5608-bp cDNA includes the entire coding region of the uterine smooth muscle MLCK.

The discovery that both the avian and mammalian smooth muscle MLCKs have conserved sequence present in their 5' noncoding regions, which corresponds to a portion of the coding region of the chicken embryo fibroblast MLCK prompted an investigation of the apparent molecular masses of MLCKs from various tissues and species. The presence of conserved sequence in the 5' noncoding region suggested that either the cDNA reported for the rabbit uterine MLCK was not a full-length cDNA, or that this region represented an exon which is included in the coding region of another form of MLCK. To clarify this issue it was necessary to determine the molecular mass of the chicken nonmuscle MLCK relative to the chicken smooth muscle MLCK. We have shown that the relative molecular masses of MLCKs are all 130 kDa in chicken gizzard, liver, and an embryo fibroblast cell line. These results are inconsistent with the expected increase in mass predicted by the chicken embryo fibroblast cDNA clone. It is possible that in primary cultures of chicken embryo fibroblasts from which the nonmuscle MLCK clone was isolated (Shoemaker et al., 1990), a larger kinase is expressed.

J Biol Chem. Author manuscript; available in PMC 2010 March 11.
that is not seen in the cell line used herein. The cDNA for the chicken fibroblast MLCK may represent a form of MLCK present early in development whereas a smaller form appears in both smooth and nonmuscle tissues later in development. Alternatively, nonmuscle tissues may express the larger MLCK at levels below the limits of detection by immunoblot, in addition to the smaller form. While the relative masses of both the smooth and nonmuscle MLCKs vary among species, it is apparent that within a given species MLCK has the same apparent molecular mass in both smooth and nonmuscle tissues. These data are consistent with other determinations of the masses of MLCKs from smooth and nonmuscle tissues. Nonmuscle MLCKs purified from bovine brain (Bartelt et al., 1987), thymus (Tawata et al., 1983), adrenal medulla (Serventi and Coffee, 1986) and stomach (Walsh et al., 1982) are reported to be 150–155 kDa, consistent with the molecular mass determined in this study for the smooth and nonmuscle MLCKs from bovine tissues. Thus, it remains to be determined which tissues or which stages in development the protein encoded by the chicken embryo fibroblast cDNA is expressed.

The highly repeated sequence motif present near the N terminus of the rabbit uterine MLCK appears to be a member of a family of repeated motifs which can be either in tandem or segmented, vary in repeat number, and length, and are rich in K, P, A, and S(T) residues (Churchill and Travers, 1991). These repeated residues are not present in avian MLCK and appear to be present in mammalian smooth and nonmuscle MLCKs. Recently, it has been proposed that repeated protein sequences which are rich in K, P, A, S (T) residues are capable of forming an unusual structural motif which might interact with DNA conformations (not specific sequences) and that within the motif a regular distribution of positively charged residues is likely to be important in stabilizing DNA-protein interactions (Churchill and Travers, 1991). Data base searches identified several proteins which have repeated sequences very similar to those present in the rabbit uterine MLCK, including DNA-binding proteins (Wells and McBride, 1989; Kato et al., 1990), and neurofilament proteins M and H (Myers et al., 1987; Lees et al., 1988; Shneidman et al., 1988). Since intermediate filament proteins bind to single-stranded DNA, neurofilament proteins may also interact with single-stranded DNA (Traub, 1985; Traub et al., 1983). It is possible that this repeat region represents a structural domain involved in the association of MLCK with chromatin. This proposal is consistent with the colocalization of MLCK to the mitotic spindle apparatus and nucleolus of interphase cells in mammalian fibroblasts (Guerriero et al., 1981).

In summary, a cDNA has been isolated which includes the entire coding region of the rabbit uterine MLCK. Information from the primary sequence deduced from this cDNA has shown that the avian and mammalian smooth muscle MLCKs are highly conserved (97% similarity) with the exception of an unusual region of repeated amino acids located near the N terminus of the rabbit kinase. It is possible that this region may represent a domain which associates with chromatin.

Acknowledgments

We wish to thank Clive Slaughter for his assistance with protein sequencing, Li Hsu for purification of chicken gizzard and bovine tracheal MLCKs and preparation of antibodies, Karen Koch for technical assistance, and Phyllis Foley for assistance in preparing this manuscript. We also wish to thank Peter McPhie (National Institute of Diabetes and Digestive and Kidney Diseases, National Institute of Health) for secondary structure analysis and suggestions concerning the repeated motif.

References

Adelstein RS, Klee CB. J Biol Chem 1981;256:7501–7509. [PubMed: 6894756]
Andersson S, Davis DL, Dahlbäck H, Jörnvall H, Russell DW. J Biol Chem 1989;264:8222–8229. [PubMed: 2722778]
Fig. 1. Construction of a full-length rabbit uterine smooth muscle MLCK

A, schematic representation of the full-length cDNA showing the relative locations of some of the restriction enzyme sites that were used to assemble the full-length molecule. The deduced coding region of the cDNA occurs between nucleotides 306 and 3746. B, Schematic representation of the three overlapping cDNA fragments obtained from a λ-gt11, oligo(dT) (top), SMPE-I (middle) and SMPE-II (bottom) primed libraries. C, schematic representation of the N- (upper, filled line) and C-terminal (middle, open line) fragments which were ligated at the XmnI site common to both constructs. The KpnI and XbaI sites originated from a pGEM polylinker and were used to ligate the 3998-bp cDNA fragment to pCMV5 generating the pCMV5-SMMLCK COS cell expression vector.
Fig. 2. Nucleotide and deduced amino acid sequence of the cDNA encoding the rabbit uterine smooth muscle MLCK

The nucleotide sequence of the full-length cDNA and the deduced amino acid sequence of coding region (uppercase letters) are shown. The 5' noncoding region has been translated and is shown in lowercase letters. Residues which are underlined are those which were determined by sequencing of peptide fragments derived from purified bovine tracheal MLCK. The nucleotides for translational initiation and termination as well as the polyadenylation signal are shown in bold letters. Nucleotides 1-14 and 5601-5608 are sequences of an adaptor and linker, respectively, which were used in construction of the cDNA libraries.

J Biol Chem. Author manuscript; available in PMC 2010 March 11.
Fig. 3. Nucleotide sequence of a portion of the rabbit smooth muscle MLCK gene
The nucleotide and deduced amino acid sequence of the 5′ portion of the rabbit smooth muscle MLCK gene are shown. Amino acids in uppercase letters are those which are also present in the rabbit uterine smooth muscle MLCK cDNA. Amino acids which are in bold and underlined are those beginning with and overlapping the translational start site predicted for the cDNA. The first bp of overlap of the genomic sequence with the cDNA sequence has been indicated by a *.
Nucleotides which are underlined are those which are proposed as a potential transcriptional start site for the rabbit smooth muscle MLCK mRNA. Nucleotides in bold are those corresponding to a primer used in the primer extension analysis.
Fig. 4. Northern analysis of RNA isolated from smooth muscle tissues

Total cellular RNA (20 μg each lane) from rabbit uterine tissue (lane 1), rat uterine tissue (lane 2), bovine tracheal tissue (lane 3) were fractionated through a 1.2% agarose gel as described in “Experimental Procedures”. The blot was probed with a 32P-labeled cDNA probe corresponding to nucleotides 574–1405 (N-terminal probe) of the full-length cDNA. Lane 4 is 5 μg of poly(A+) mRNA from rabbit uterine tissue which was probed with a 32P-labeled cDNA probe corresponding to nucleotides 3350–3998 (C-terminal probe) of the full-length cDNA. The positions of the RNA molecular weight standards are shown in kilobases on the left and an arrowhead on the right side corresponds of the position of the 5.8-kb mRNAs. The
lanes representing the rabbit uterine tissue (lane 1 and lane 4) were exposed for 24 h at −70 °C, all other lanes were exposed for 72 h at −70 °C.
Fig. 5. Immunoblot analysis of MLCK

A, immunoblot analysis of recombinant smooth muscle MLCK expressed in COS cells. Lysates from COS cells transfected with either pCMV5 (mock), pCMV5-SMMLCK or homogenates from bovine tracheal or rabbit uterine smooth muscle tissue were prepared as described under “Experimental Procedures.” Lane 1, 2 ng of purified bovine tracheal muscle MLCK; lane 2, 4 μg of bovine tracheal tissue extract; lane 3, 39 μg of total protein rabbit uterine tissue extract; lane 4, 30 μl of COS cell lysate from cells transfected with pCMV5-SMMLCK; lane 5, 30 μl of COS cell lysate from cells transfected with pCMV5(mock).

B, immunoblot analysis of smooth and nonmuscle rabbit, chicken and bovine tissues. Tissue extracts and immunoblots were prepared as described in “Experimental Procedures.” The amount of total protein loaded per lane varies as indicated and is not representative of relative amounts of immunoreactive protein present in these tissues. Rabbit uterus (39 μg, lane 1), rabbit trachea (178 μg, lane 2), rabbit aorta (86 μg, lane 3), rabbit ileum (83 μg, lane 4), rabbit kidney (168 μg, lane 5), rabbit adrenal (220 μg, lane 6), and rabbit uterus (39 μg, lane 7); chicken gizzard (12 μg, lane 8), chicken liver (38 μg, lane 9); bovine trachea (4 μg, lane 10), bovine adrenal (13 μg, lane 11) and purified bovine tracheal MLCK, 2 ng (lane 12).

C, immunoblot analysis of cultured cells. Lane 1, chicken gizzard tissue (12 μg of total protein); lane 2, 60 μl of cell lysate from chicken embryo fibroblast cells. The smaller molecular weight immunoreactive bands appearing in lane 1 are degradative fragments of the chicken gizzard MLCK. The positions of the molecular mass standards are indicated on the right side of the figures and apparent molecular masses for MLCKs from these tissues are summarized in Table II.
Fig. 6. Amino acid alignment of chicken nonmuscle, chicken smooth muscle, and rabbit smooth muscle MLCKs

Residues 239–972 of the chicken embryo fibroblast nonmuscle (CKNM, top), 1–972 of the chicken gizzard smooth muscle (CKSM, middle), and 1–1147 of the rabbit uterine smooth muscle MLCK (RBSM, bottom) have been aligned for maximum homology. Residues which are identical and similar in all three sequences have been indicated by a * and ., respectively, below the alignment. Residues contained within the catalytic core and regulatory/calmodulin-binding domains of the enzymes are in bold letters (Olson et al., 1990).
Fig. 7. Alignment of the repeated amino acid sequence within the N terminus of the rabbit smooth muscle MLCK.

The KP (A/V) amino acids are represented in **bold letters**.

| T | L | K | F | V | A | N | T | K | P | A | E |
|---|---|---|---|---|---|---|---|---|---|---|---|
| T | L | K | P | V | A | N | A | E |
| T | L | K | P | M | G | N | A | K | P | A | E |
| S | S | K | F | V | G | N | T | K | P | A | E |
| T | L | K | P | V | G | N | T | K | P | A | E |
| T | L | K | P | V | G | N | I | K | P | A | E |
| T | L | K | P | V | G | N | I | K | P | A | E |
| T | L | K | P | V | G | N | T | K | P | T | E |
| T | L | K | P | V | A | N | A | K | S | A | E |
| T | L | K | P | I | A | N | T | K | P | A | E |
| T | L | K | P | V | G | N | A | K | P | A | E |
| T | L | K | P | V | G | N | A | K | P | A | E |
| T | L | K | P | V | G | N | A | K | P | A | E |
| T | L | K | P | V | G | N | A | K | P | A | E |
### Table I

**Enzymatic activity of purified and COS cell expressed smooth muscle MLCKs**

The kinase activity and kinetic constants of the expressed kinase was determined as described under “Experimental Procedures.” The values represent means ± S.E. for at least four experiments.

| Protein                                    | $V_{max}^a$ | $K_m^b$ |
|--------------------------------------------|-------------|---------|
| Purified chicken gizzard MLCK$^c$          | 36.2 ± 1.6  | 8.6 ± 1.5 |
| Recombinant rabbit uterine smooth muscle   | 39 ± 5      | 9 ± 1.2  |

$^a$ The units for activity are pmol $^{32}$P incorporated/min · ng enzyme.

$^b$ Light chain from chicken gizzard.

$^c$ Kemp et al. (1983).
Table II
Summary of the relative molecular masses of smooth and nonmuscle MLCKs

| Species  | Smooth muscle MLCK\(^b\) | Nonmuscle MLCK\(^c\) |
|----------|--------------------------|----------------------|
| Bovine   | 155                      | 155                  |
| Rabbit   | 152                      | 152                  |
| Chicken  | 130                      | 130                  |
| Rat      | 136                      | N.D.                 |

\(^a\) Values reported in kDa.

\(^b\) Representative smooth muscle tissues included uterus, trachea, aorta, ileum, and gizzard.

\(^c\) Representative nonmuscle tissues included kidney, liver, and adrenal.

N.D. = not determined.
Table III
Repeated protein motifs which are analogous to those present near the N terminus of the rabbit uterine MLCK

| Protein                                      | Repeat unit  | Type    | Reference          |
|----------------------------------------------|--------------|---------|--------------------|
| Rabbit MLCK                                  | TLKPVANAKPÆ₁₅| Tandem  | This report        |
| Histone H₁ (sea urchin)                      | KPA₁₋₂K      | Segmented | Wells and McBride (1989) |
| AlgR₃ (Pseudomonas aeruginosa)                | KPA₂₁₀       | Tandem  | Kato et al. (1990)  |
| Neurofilament-M (human)                      | KSPVPKSPVEEK₉₈ | Tandem  | Myers et al. (1987) |
|                                              | KSPV₁₃       | Segmented|                   |
| Neurofilament-H (human)                      | KSPEKAKSPVKAÆ₂₃₉ | Segmented | Loes et al. (1988) |
|                                              | KSP₄₂        | Segmented|                   |
| Neurofilament-H (mouse)                      | KSP₄₈Æ(A,P,V)₁₅₇ | Tandem  | Shneidman et al. (1988) |

*a* The consensus repeat unit is shown; residues which are underlined are subrepeat units which are repeated within the consensus repeat unit.