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Characterization and Differential Expression of Human Vascular Smooth Muscle Myosin Light Chain 2 Isoform in Nonmuscle Cells†

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Received October 25, 1988; Revised Manuscript Received January 13, 1989

ABSTRACT: The 20-kDa regulatory myosin light chain (MLC), also known as MLC-2, plays an important role in the regulation of both smooth muscle and nonmuscle cell contractile activity. Phosphorylation of MLC-2 by the enzyme MLC kinase increases the actin-activated myosin ATPase activity and thereby regulates the contractile activity. We have isolated and characterized an MLC-2 cDNA corresponding to the human vascular smooth muscle MLC-2 isoform from a cDNA library derived from umbilical artery RNA. The translation of the in vitro synthesized mRNA, corresponding to the cDNA insert, in a rabbit reticulocyte lysate results in the synthesis of a 20 000-dalton protein that is immunoreactive with antibodies raised against purified chicken gizzard MLC-2. The derived amino acid sequence of the putative human smooth muscle MLC-2 shows only three amino acid differences when compared to chicken gizzard MLC-2. However, comparison with the human cardiac isoform reveals only 48% homology. Blot hybridizations and S1 nuclease analysis indicate that the human smooth muscle MLC-2 isoform is expressed restrictively in smooth muscle tissues such as colon and uterus and in some, but not all, nonmuscle cell lines. Previously reported MLC-2 cDNA from rat aortic smooth muscle cells in culture is ubiquitously expressed in all muscle and nonmuscle cells, and it was suggested that both smooth muscle and nonmuscle MLC-2 proteins are identical and are probably encoded by the same gene. In contrast, the human smooth muscle MLC-2 cDNA that we have characterized from an intact smooth muscle tissue is not expressed in skeletal and cardiac muscles and also in a number of nonmuscle cells. Nevertheless, MLC-2 protein species is readily detectable in all the nonmuscle cell lines using antibodies to smooth muscle MLC-2 protein. Two-dimensional gel analysis of the ^{35}S-labeled proteins from a nonmuscle cell line indicates three protein species that are immunoprecipitated with MLC-2 antibodies. Comparison of the two-dimensional gel pattern indicates the absence of one MLC-2 protein species in the cell lines that do not express the smooth muscle MLC-2 mRNA. Together, these results suggest that the smooth muscle and nonmuscle MLC-2 isoforms are separate and are possibly encoded by separate genes. Hence, the MLC-2 cDNA sequence reported in this paper corresponds to a novel and distinct smooth muscle isoform.

The two major contractile proteins actin and myosin are present in almost all eukaryotic cells. Myosin is a hexameric protein which forms the core of the thick filaments of muscle. All myosin filaments share the same architecture of two myosin heavy chains (MHCs) and two pairs of light chains (MLCs). Two of these light chains are classified as phosphorylatable regulatory chains (MLC-2), and the other two are nonphosphorylatable, alkali light chains (MLC-1 or MLC-3) (Harrington & Rodgers, 1984). Myosin light chain 2 (MLC-2) has a molecular weight of 20K and plays an important role in the regulation of smooth muscle contraction

† The nucleic acid sequence in this paper has been submitted to GenBank under Accession Number J02854.
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0006-2960/89/0428-4027$01.50/0

(Adelstein & Eisenberg, 1980; Hartshorne, 1982).

MLC-2 is phosphorylated by the enzyme myosin light chain kinase (MLCK) in the presence of calcium and calmodulin (Walsh & Hartshorne, 1982; Adelstein & Klee, 1981). This phosphorylation event has been found to increase actin-activated ATPase activity of both smooth muscle and nonmuscle myosins (Chako et al., 1977; Sobieszek & Small, 1977). It is generally accepted that phosphorylation of MLC-2, in smooth and nonmuscle tissues, is involved in the regulation of contraction (Adelstein, 1983). Early work with in vitro systems identified serine-19 as the phosphorylation site by the enzyme MLCK (Kemp et al., 1983; Kemp & Pearson, 1985). Later work showed that threonine-18 was also phosphorylated by the same enzyme (Ikebe et al., 1986). This second site of phosphorylation also increases actin-activated ATPase activity. MLC-2 is also phosphorylated at other sites by a number of enzymes, as well as epidermal growth factor (Gallis et al., 1983) and protein kinase C (Nishikawa et al., 1984). However, the implications of these phosphorylations are not well understood.

Structurally related MLC-2 protein isoforms exhibit tissue-specific expression and are encoded by multigene families (Siddiqui & Kumar, 1987). Both skeletal and cardiac muscle MLC-2s share strong regions of homology with smooth muscle MLC-2 amino acid sequences flanking the phosphorylated serine residue (Pearson et al., 1984). However, phosphorylation does not result in the dramatic changes in ATPase activity and does not appear to be necessary for skeletal or cardiac muscle contraction (Harrington & Rodgers, 1984). Thus, MLC-2 plays a unique role in smooth muscle and nonmuscle tissues. The primary structure of MLC-2, thus, is believed to contribute to this unique role. In addition, the regulation of the expression of smooth muscle MLC-2 gene(s) may also play an important role in regulating smooth muscle contraction.

It is generally accepted that smooth muscle cell proliferation and migration from the arterial media into the intima are the essential steps in the development of the atheromatous plaque (Ross, 1986). Very little is known about the biochemical differences between medial smooth muscle cells and the smooth muscle cells of the atheromatous plaque. A MLC-2 cDNA clone derived from rat aortic smooth muscle cells in culture has been characterized recently (Taubman et al., 1987). This rat aortic MLC-2 gene was expressed in all muscle and nonmuscle tissues, and hence the suggestion was made either that the cDNA characterized encodes the nonmuscle MLC-2 or that the nonmuscle and smooth muscle MLC-2s are products of the same gene or of genes with similar coding regions (Taubman et al., 1987). It is very well established that smooth muscle cells in culture express both contractile and noncontractile isoforms (Chamley-Campbell et al., 1979), and evidence for the presence of multiple MLC-2 isoforms in rat aortic smooth muscle cells in culture has been obtained (Kawamoto & Adelstein, 1988; Csabina et al., 1986). Hence, it is not clear that the rat aortic MLC-2 cDNA corresponds to an authentic smooth muscle isoform. In order to gain a better understanding of the primary structure of the vascular smooth muscle MLC-2 and to analyze its expression in other smooth muscle tissues and in atheromatous plaques, we have undertaken the cloning and characterization of MLC-2 cDNA derived from human vascular smooth muscle tissue instead of cells in culture. Contrary to the observations made using rat aortic MLC-2 cDNA, our results indicate that the smooth muscle MLC-2 isoform is not expressed in all muscle and nonmuscle cells and that nonmuscle cells express more than one MLC-2 isoform, indicating that smooth muscle and nonmuscle MLC-2 isoforms are distinct and are possibly encoded by separate genes.

Materials and Methods

Reagents. Restriction endonucleases, DNA-modifying enzymes, and the M13 sequencing kit were purchased from New England Biolabs, Boston, MA. Double-stranded M13 mp9 vector was obtained from New England Nuclear Corp. Radioisotopes and cDNA synthesis system were purchased from Amersham Corp., Arlington Heights, IL. λ packaging extracts and λgt-10 arms were purchased from Promega Corp., Madison, WI.

Isolation of RNA. Human umbilical cords were obtained from St. Joseph's Hospital in Paterson, NJ. Arteries were excised and stored frozen in liquid N_2. Normal human colon and uterine smooth muscle tissues obtained from Sloan-Kettering Cancer Center in New York were stored frozen in liquid N_2. Total RNA was extracted from frozen tissues by homogenization in 4 M guanidinium thiocyanate as described by Chirgwin et al. (1979). The homogenate was layered on 5.7 M cesium chloride solution containing 0.1 M EDTA, pH 7.0, and centrifuged at 100000*g* for 20 h at 15 °C. The RNA pellet was suspended in 0.1 M Tris-HCl, pH 7.6, 50 mM NaCl, 10 mM EDTA, 1% SDS, and 100 μg/mL proteinase K. After incubation at 37 °C for 30 min, RNA was recovered by phenol and chloroform extraction followed by ethanol precipitation. Poly(A+) RNA was purified from total RNA by two cycles of affinity chromatography on oligo(dT)–cellulose (Aviv & Leder, 1972).

Construction and Screening of Human Vascular Smooth Muscle Derived cDNA Library. Double-stranded cDNA was synthesized following the procedures of Gubler and Hoffman (1983) using 10 μg of poly(A+) RNA isolated from umbilical artery.

The cDNA library consisting of 10^5 recombinant phages was screened with a nick-translated cDNA fragment corresponding to the rat aortic smooth muscle MLC-2 by using procedures described by Young and Davis (Huyan et al., 1984). Four positive plaques were identified by 4 rounds of screening from the library of 100 000 recombinants. The plaques were amplified and used for large-scale preparation of phage DNA. The cDNA inserts were isolated from the λgt-10 clones by digestion with *Eco*RI endonuclease followed by electrophoresis on a 0.8% agarose gel. The inserts were subcloned into M13 mp9 phage vector, and single-stranded DNA was prepared (Messing et al., 1981).

Antiserum Preparation. The rabbit antiserum made against purified chicken gizzard myosin light chain 2 was a generous gift of Drs. Meeta Chatterjee and Carolyn Foster of the Cardiovascular Division, Schering Research. MLC-2 was purified from chicken gizzard tissue by following the procedure described by Bartelt et al. (1987), and antibodies were raised in rabbit by following standard procedures (Bartelt et al., 1987). A 1:500 dilution of the antiserum was used for immunoprecipitation experiments.

In Vitro Transcription and Translation. For in vitro transcription, the 1.1-kb cDNA insert from phage λHu MLC-6 was subcloned into pGEM-4 vector at the *Eco*RI site. The plasmid DNAs containing the cDNA insert in either orientation were linearized by cutting with appropriate restriction enzymes and used as templates in the in vitro transcription reactions using SP6 RNA polymerase according to the conditions suggested by the manufacturer (Promega Biotec). The transcription mixture was treated with DNase I, and the transcripts were recovered by phenol extraction and

ethanol precipitations. One to two micrograms of in vitro transcripts was added to 35 μL of rabbit reticulocyte lysate (Promega Biotec) in the presence of [^{35}S]methionine. Following incubation at room temperature for 30 min, 2 μL of translation mixture was loaded onto a 12.5% reducing SDS–polyacrylamide gel, and the products were visualized by fluorography. For immunoprecipitation, 2 μL of the lysate was diluted into 400 μL of Triton buffer containing 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 10 mM Tris-HCl, pH 7.2, and 1 mM phenylmethanesulfonyl fluoride (PMSF). MLC-2 antiserum (5 μL) was added to the diluted lysate. After 2 h on ice, protein A–agarose (Bio-Rad) was added and incubated for 30 min on ice and then pelleted by centrifugation. After being washed 3 times with the Triton buffer, the pellet was resuspended in a small volume of 3% SDS/10% β-mercaptoethanol and heated to 100 °C for 5 min to dissociate the immune complexes. The supernatant was then removed, lyophilized, and resuspended in sample buffer for gel electrophoresis.

Nucleic Acid Sequence Determination. DNA sequence determination was performed by the dideoxy sequencing method of Sanger (Sanger et al., 1977). Sequences at the ends of the cloned fragments in M13 were determined by using commercially available universal M13 pentadecamer primer. The sequences of the inserts were completed by synthesizing appropriate oligonucleotides for use as primers in further sequencing experiments. The oligonucleotides of 15 bases in length were synthesized by using the Applied Biosystems 380A DNA synthesizer. Primer concentrations were estimated by measurement of OD_{260} and diluted in water at 0.8 pmol/μL, and 1 μL of the diluted oligonucleotide was used in each sequencing reaction.

RNA Blot Hybridization. Total RNA (10 μg) was size fractionated by electrophoresis on 1% agarose gels in 200 mM 4-morpholinepropanesulfonic acid (MOPS), pH 7.4, 1 mM EDTA, and 3% formaldehyde. Transfer to nitrocellulose (Schleicher & Schuell) and hybridization to nick-translated ^{32}P-labeled DNA were as described (Maniatis et al., 1982). At the end of hybridization, the filters were washed twice at room temperature in 2× SSC/0.1% SDS and twice in 0.2× SSC/0.1% SDS at 55 °C. Autoradiography was at −70 °C with preflashed film and an intensifying screen.

S1 Nuclease Protection Analysis. Uniformly labeled single-stranded (ss) DNA probes were synthesized for S1 protection assay (Burke, 1984). The ssDNA was isolated from the phage with the desired orientation of the cDNA insert and used as a template for primer extension using the M13 universal primer. After restriction with *Hin*dIII restriction enzyme, the radiolabeled ssDNA probe was purified on a 5% polyacrylamide/8 M urea gel. For hybridization, approximately 10 000 cpm of the probe were incubated with 20 μg of total RNA at 37 °C for 16–20 h in 10 μL of hybridization buffer containing 80% formamide, 400 mM EDTA, and 40 mM PIPES (pH 6.4). Hybridizations were diluted with 300 μL of S1 digestion buffer consisting of 280 mM sodium chloride, 30 mM sodium acetate, pH 4.4, 4.5 mM zinc acetate, 20 μg of denatured salmon sperm DNA, and 1000 units of S1 nuclease (Boehringer-Mannheim). Following incubation at 37 °C for 1 h, the hybrids were recovered by phenol extraction and ethanol precipitation. Electrophoresis was on thin 5% acrylamide–urea gels after denaturation with formamide dye by boiling for 3 min.

Cell Culture. HL-60 and Jurkat leukemic T cells, obtained from American Tissue Culture Collection, were cultured in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum, glutamine (2 mM), β-mercaptoethanol (0.03%), penicillin (50 units/mL), and streptomycin (50 μg/mL). Activated T cell cultures were prepared as described by Rusceti et al. (1977) from human peripheral blood. Briefly, heparinized whole blood was obtained from donors to prepare a mononuclear cell suspension using the Ficoll–Hypaque method (Boyum, 1968). The macrophages were removed by allowing them to adhere to a plastic dish, and the nonadherent cells were washed twice and resuspended in RPMI 1640 culture medium. These cultures (5×10^6 cells/mL) were incubated at 37 °C in a 5% CO_2-humidified atmosphere in the presence of phytohemagglutinin (1 μg/mL) for 2 days to induce continuous T-cell growth. Human diploid fibroblast cell line KD obtained from Dr. John Leavitt (Linus Pauling Institute, Palo Alto, CA) was cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum. RNA was isolated from these cell lines by a modified guanidinium thiocyanate procedure as described by Cathalia et al. (1983).

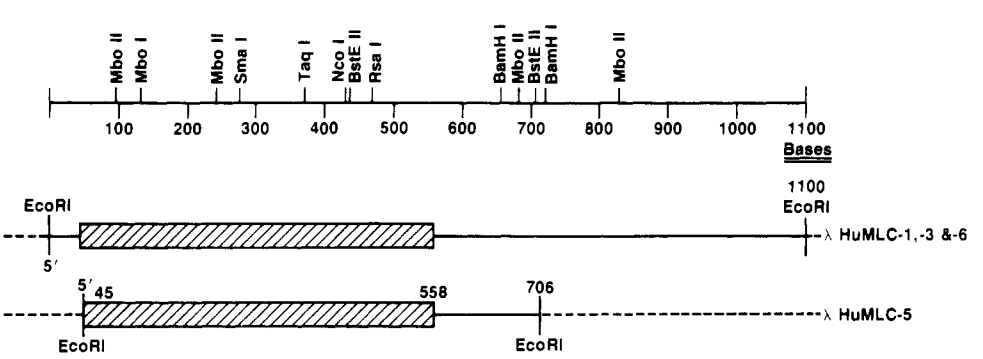
Two-Dimensional Gel Electrophoresis. Lysates for two-dimensional gel analysis were prepared from cells labeled for 16 h with 500 μCi/mL [^{35}S]methionine (Amersham) in 3 mL of methionine-free DMEM medium supplemented with 2.5% fetal calf serum. The cells were lysed with 0.3% SDS containing 1% β-mercaptoethanol and 20 mM Tris, pH 8.0, preheated to 100 °C. The two-dimensional gels were run and analyzed at the QUEST gel lab facility at Cold Spring Harbor, NY (Garrels, 1983).

Lysates for immunoprecipitation were prepared by addition of 1% Triton/1% deoxycholate in phosphate-buffered saline (PBS), pH 7.3, to labeled cells. After 15 min on ice, the cells were scraped from the dish and passed 5 times through a 28-gauge needle, and protein A–Sepharose was added. After 30 min, the protein A–Sepharose was removed by centrifugation, and the specific antibody was added to the supernatant. After 2 h on ice, protein A–Sepharose was added, and the supernatant was incubated for 30 min on ice and then pelleted by centrifugation. After being waxed 3 times with the above buffer and 1 time with PBS, the pellet was resuspended in a small volume of 3% SDS/10% β-mercaptoethanol and heated to 100 °C for 5 min to dissociate the immune complexes. The supernatant was then removed, lyophilized, and resuspended in sample buffer for two-dimensional electrophoresis.

Results

Isolation and Characterization of Human Vascular Smooth Muscle MLC-2 cDNA Clones. A cDNA library consisting of 1×10^5 recombinants was constructed in λgt-10 vector using 10 μg of poly(A+) RNA isolated from human umbilical arteries. The cDNA library was screened with a rat aortic smooth muscle MLC-2 cDNA insert, obtained from Dr. Taubman (Peter Brigham Hospital, Boston, MA). It is known that MLCs within one tissue type share extensive amino acid sequence homology in many species (Siddiqui & Kumar, 1987). Screening of the library under medium stringency of washing conditions (2× SSC and 0.1% SDS at 55 °C) resulted in four positive clones which were purified after four rounds of repeated plating and screening. Three of these clones (HuMLC-1, HuMLC-3, and HuMLC-6) had inserts of about 1100 base pairs (bp) in length, and the other one (HuMLC-5) carried an insert of about 700 bp. Southern blot analysis confirmed that all the inserts specifically hybridized to the nick-translated rat aortic smooth muscle MLC-2 cDNA insert. These inserts were excised from λgt-10 DNA and subcloned into M13 mp9 phage vector. Preliminary restriction endonuclease mapping demonstrated that all clones were derived

a)



b)

```
5'
ACT TCT TCG CAC CAG GGA AGC CCC ACC CAC CAG AAC GCC AAG ATG TCC AGC AAG CGG GCC AAA GCC AAG      69
                                                        MET Ser Ser Lys Arg Ala Lys Ala Lys
                                                         1

GCC ACC AAG AAG CGG CCA CAG CGG GCC ACA TCC AAT GTC TTC GCA ATG TTT GAC CAG TCC CAG ATC CAG     138
Thr Thr Lys Lys Arg Pro Gln Arg Ala Thr Ser Asn Val Phe Ala MET Phe Asp Gln Ser Gln Ile Gln
 10                                  20                                  30

GAG TTT AAG GAG GCT TTC AAC ATG ATT GAC CAG AAC CGT GAT GGC TTC ATT GAC AAG GAG GAC CTG CAC     207
Glu Phe Lys Glu Ala Phe Asn MET Ile Asp Gln Asn Arg Asp Gly Phe Ile Asp Lys Glu Asp Leu His
                            40                                  50

GAC ATG CTG GCC TCG CTG GGG AAG AAC CCC ACA GAC GAA TAC CTG GAG GGC ATG ATG AGC GAG GCC CCG     276
Asp MET Leu Ala Ser Leu Gly Lys Asn Pro Thr Asp Glu Tyr Leu Glu Gly MET MET Ser Glu Ala Pro
                  60                                  70

GGG CCA TAC AAC TTC ACC ATG TTC CTC ACC ATG TTT GGG GAG AAG CTG AAC GGC ACG GAC CCC GAG GAT     345
Gly Pro Tyr Asn Phe Thr MET Phe Leu Thr MET Phe Gly Glu Lys Leu Asn Gly Thr Asp Pro Glu Asp
 80                  90                                                  100

GTG ATT CGC AAC GCC TTT GCC TGC TTC GAC GAG GAA TCC TCA GGT TTC ATC CAT GAG GAC CAC CTC CGG     414
Val Ile Arg Asn Ala Phe Ala Cys Phe Asp Glu Glu Ser Ser Gly Phe Ile His Glu Asp His Leu Arg
                        110                                 120

AAG CTG CTC ACC ACC ATG GGT GAC CGC TTC ACA GAT GAG GAA GTG GAC GAG ATG TAC CGG GAG GCA CCC     483
Glu Leu Leu Thr Thr MET Gly Asp Arg Phe Thr Asp Glu Glu Val Asp Glu MET Tyr Arg Glu Ala Pro
                    130                                 140

GTT GAT AAG AAA GGC AAC TTC AAC TAC GTG GAG TTC ACC CGC ATC CTC AAA CAT GGC GCC AAG GAT AAA     552
Ile Asp Lys Lys Gly Asn Phe Asn Tyr Val Glu Phe Thr Arg Ile Leu Lys His Gly Ala Lys Asp Lys
          150                                 160                                 170

CAC GAC TAG GCC ATC CCC AGC CCC CTG ACA CCC AGC CCC CGC CAG TCA CCC CTC CCC GCA CAC ACC CGT     621
Asp Asp.
     End
     172


CCA TAC CAG CTC CCT GCC CAT GAC CCT CGC TCA GGG ATC CCC CTT TGA GGG TTA GGG TCC CAG TTC CCA     690

GTG GAA GAA ACA GGC CAG GAG AGT GCG TGC CGA GCT GAG GCA GAT GTT CCC ACA GTG ACC CCA GAG CCC     759

TGG GCT ATA GTC TCT GAC CCC TCC AAG GAA AGA CCA CCT TCT GGG GAC ATG GGC TGG AGG GCA GGA CCT     828

AGA GGC ACC AAG GGA ACC GCA TTC CGG GGC TGT TCC CCG AGG AGG AAG GGA AGC CTC TGT GTG CCC CCC     897

AGG AGG AAG AGG CCC TGA GTC CTG GGA TCA GAC ACC CCT TCA CGT GTA TCC CAC ACA AAT GCA AGC TCA     966

CCA AGG TCC CCT CTC AGT CCC CTT CCC TAC ACC CTG ACG CCA GAT GCC GCA CAC CCA ACG CCA CCA GCC    1035

ATG GGA GTG TGC TCA GGA GTC GCG GGG CAG ACG TGA CAT CTG TCC AGA GGG GGC AGA ATC TCC AAT AGA    1104

GGA CTG AGA CAA CAT GAAAAAAAAAAAA                                                            1132
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FIGURE 1: Physical map of the two types of recombinant λgt-10 phages encoding MLC-2 cDNA insert (a) and the nucleotide and derived amino acid sequences of the full-length cDNA insert (b). (a) The scale at the top is in base pairs, arbitrarily assigning a value of 1 to the 5'-most nucleotide in the insert of clone HuMLC-1 (excluding the *Eco*RI linker). The two types of phages, obtained by screening the human umbilical artery cDNA library with rat aortic MLC-2 cDNA probe, are aligned with respect to one another. The hatched boxes represent the coding region of the cDNA inserts. The numbers on top indicate the exact position of the smaller cDNA insert in HuMLC-5, which is also shown in the sequence with arrows. (b) The nucleotide and deduced amino acid sequence of the larger cDNA insert (HuMLC-6) are shown. Arrows indicate the boundaries of the smaller cDNA insert in λHuMLC-6. Potential polyadenylation signal is underlined.

from the same mRNA and the 700-bp insert is a partial cDNA clone. The complete nucleotide sequences of both the 700-bp and the 1.1-kb inserts were obtained by using the dideoxy chain-termination method of Sanger et al. (1977). Specific oligonucleotide primers of 15 bases in length were synthesized and used to obtain overlapping sequence information of the cDNA inserts. Figure 1 shows the nucleotide sequence and derived amino acid sequence for the putative vascular smooth muscle MLC-2 cDNA. The cDNA insert contains 42 nucleotides of 5'-untranslated region, a 516-nucleotide-long (172 amino acids) coding region, and 572 bases of the 3'-untranslated region ending in a short 16-nucleotide-long poly(A) tail. The 3'-untranslated region is very rich in GC clusters, and it is interesting to note that the smooth muscle MLC-2 mRNA contains very long 3'-untranslated region, compared to both the skeletal and cardiac isoforms (Kumar et al., 1986;

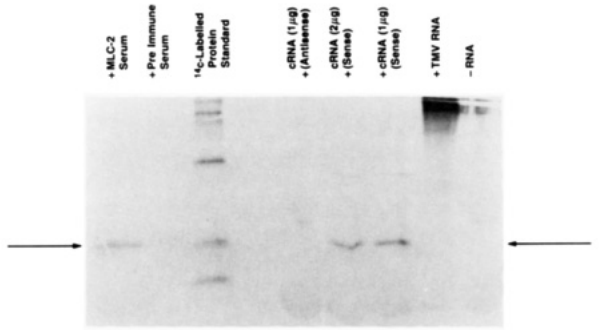

FIGURE 2: In vitro transcription and translation of the MLC-2 cDNA. Reticulocyte lysates programmed with in vitro transcripts in the presence of [^{35}S]methionine were analyzed using a SDS–polyacrylamide gel. In vitro transcripts were generated by SP6 RNA polymerase using pGEM plasmid templates containing the HuMLC-6 cDNA insert in both sense and antisense orientations. Tobacco mosaic virus (TMV) RNA was used as a control to follow the efficiency of lysate protein synthesis. A portion of the reticulocyte lysate translation system programmed with MLC-2 mRNA was immunoprecipitated with MLC-2 antibodies as described under Materials and Methods, and the immunoprecipitates were analyzed on the same gel. The arrows denote the mobility of the 20-kDa MLC-2 protein species. ^{14}C-Labeled protein size markers, bovine serum albumin (66K), ovalbumin (45K), carbonic anhydrase (31K), and cytochrome *c* (12K), were used. Gels were processed for fluorography using Enhance (NEN) as suggested by the manufacturers.

Nudel et al., 1984). The importance of this long 3′-untranslated region with very high GC content is not known.

The cDNA insert from phage λHu MLC-1 was subcloned into pGEM4 vector containing the SP6 promoter. The in vitro synthesized cRNA, using SP6 RNA polymerase, directs the synthesis of a protein of 20 000 molecular weight in rabbit reticuloycte lysate (Figure 2). This protein is also immunoprecipitated with antibodies raised against purified chicken gizzard MLC-2. These results further confirm that the cDNA clone does encode an MLC-2 isoform.

Figure 3 shows a comparison of the derived amino acid sequence of the human vascular smooth muscle MLC-2 with published amino acid sequences of MLC-2s derived from chicken gizzard (Pearson et al., 1984), rat aortic smooth muscle (Taubman et al., 1987), and human cardiac (Klotz et al., 1982) tissues. Only partial amino acid sequence data were available for the human cardiac isoform. It can be seen that the MLC-2 expressed in smooth muscle tissues is highly conserved between species. From amino acid 1 to 171, there are only 3 and 13 differences between human vascular smooth muscle and chicken gizzard and rat aortic MLC-2, respectively. However, comparison with the human cardiac isoform, even with the partial sequence data, shows only 48% homology. Thus, the smooth muscle MLC-2s of two different species share significantly higher homology than the isomers originating in different muscle tissues of the same species. Despite the very high homology of the amino acid sequences in these proteins, there is less homology at the nucleotide sequence level due to divergence of the sequences in the noncoding segments. The sequence derived from human vascular smooth muscle MLC-2 cDNA is in agreement with the chicken gizzard MLC-2 protein sequence published by Pearson et al. (1984).

Tissue Specificity and Expression of MLC-2 mRNA in Smooth Muscle and Nonmuscle Tissues. The contractile protein genes of skeletal and cardiac muscle are expressed in a developmental and tissue-specific manner (Siddiqui & Kumar, 1987). Changes in the expression of human cardiac MLC-2 isoforms in response to increased pressure load and subsequent hypertrophy have been observed (Cummins, 1983). Previously reported rat aortic MLC-2 has been shown to be

```
CG     SSKRAKAKTT KKRPQRATSN VFAMFDQSQI QEFKEAFNMI DQNRDGFIDK EDLHDMLASM      60
HVSM   ---------- ---------- ---------- ---------- ---------- --------L
RASM   ------T--- ---------- ---------- ---------- ---------- --------L
HC     .......... ....A---.- --S*-E.T-- -------TVM ---------- N--R-TF-A.

CG     GKNPTDEYLE GMMSEAPGPI NFTMFLTMFG EKLNGTDPED VIRNAFACFD EEASGFIHED     120
HVSM   ---------- ---------Y ---------- ---------- ---------- --S-------
RASM   ---------D A--N------ ---------- ---------- ---------- ---I-T-Q--
HC     .......... .......... .........-- ---K-A---E T-L---KVL- D-GK-IEADY

CG     HLRELLTTMG DRFTDEEVDE MYREAPIDKK GNFNYVEFTR ILKHGAKDKD D              171
HVSM   ---------- ---------- ---------- ---------- ----------
RASM   Y--------- ---------- L--------- -----I---- ----------
HC     IK...---QA .-**SK-.-Q -FAAF-P-VT --LD-KNLLH -IT--EGK.

       CG:    CHICKEN GIZZARD MLC-2
       HVSM:  HUMAN VASCULAR SMOOTH MUSCLE MLC-2
       RASM:  RAT AORTIC SMOOTH MUSCLE MLC-2
       HC:    HUMAN CARDIAC MLC-2
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FIGURE 3: Comparison of the predicted human smooth muscle MLC-2 protein sequence with other published MLC-2 sequences. Amino acid sequence comparison with rat aortic smooth muscle MLC-2 (RASM, ref 21), chicken gizzard smooth muscle MLC-2 (CG; Pearson, 1984), and human cardiac muscle MLC-2 (HC; Klotz, 1982) are shown. Numbering on the human vascular smooth muscle MLC-2 sequence, with the initiating methionine absent. A dash represents amino acids identical with that of HVSM MLC-2. An asterisk is used to denote positions where amino acids are not present. A dot is used to denote an undetermined amino acid sequence.

expressed in a large variety of muscle and nonmuscle tissues, except liver (Taubman et al., 1987). To examine the expression of the gene encoding the human smooth muscle MLC-2 mRNA represented by the cDNA clone described here, RNA blot analysis was undertaken using total RNA isolated from uterus and colon smooth muscle tissues, cardiac and skeletal muscle tissues, and a nonmuscle cell such as human foreskin fibroblasts. Figure 4 shows a typical experiment in which the various RNAs were hybridized with the ^{32}P-labeled 1.1-kb cDNA insert from clone HuMLC-6. Filters were washed at a high stringency of washing conditions (0.1× SSC/0.1% SDS at 65 °C). A hybridizing species migrating at approximately 1 kb in length can be observed in the smooth muscle tissues examined. This species has the expected size for the putative smooth muscle MLC-2. The hybridizing RNA species is not detectable in cardiac and skeletal muscle tissues. Rehybridization of the same blot with human β-actin cDNA probe verified the integrity of the RNA preparations (data not shown).

To further examine this phenomenon and to get a better understanding of the nature of the hybridizing species seen on the RNA blots, S1 nuclease analysis was performed using RNAs derived from the same tissues and cell lines as described above. Uniformly labeled single-stranded DNA probes were synthesized by using M13 mp9 single-strand virion DNA containing human smooth muscle MLC-2 cDNA insert in the desired orientation. The phage DNA was used as a template for primer extension using the M13 universal primer (NEB) and DNA polymerase I large fragment. The primer-extended probe contains about 50 nucleotides of the M13 DNA. Hence, the fully protected species after S1 nuclease digestion following hybridization to the total RNA is shorter than the probe, since the M13 DNA portion is cleaved by S1 nuclease. As can be seen in Figure 4c, the S1 nuclease mapping experiment demonstrates the presence of RNA homologous to the human vascular smooth muscle MLC-2 cDNA in colon and uterus smooth muscle as well as in foreskin fibroblasts cells in culture but not in human cardiac muscle tissue. To examine the expression of the smooth muscle MLC-2 isoform in other nonmuscle cells, we isolated RNA from human diploid fibroblast cell lines such as KD, WI38 (human lung fibroblast), foreskin fibroblasts, and also a number of hemopoietic cells such as activated T cells derived from three normal individuals, Jurkat (a leukemic T cell line), and the HL-60 (a promyelomonocytic cell line). Northern blot analysis (Figure 5) clearly shows that the MLC-2 isoform is expressed in the fibroblast

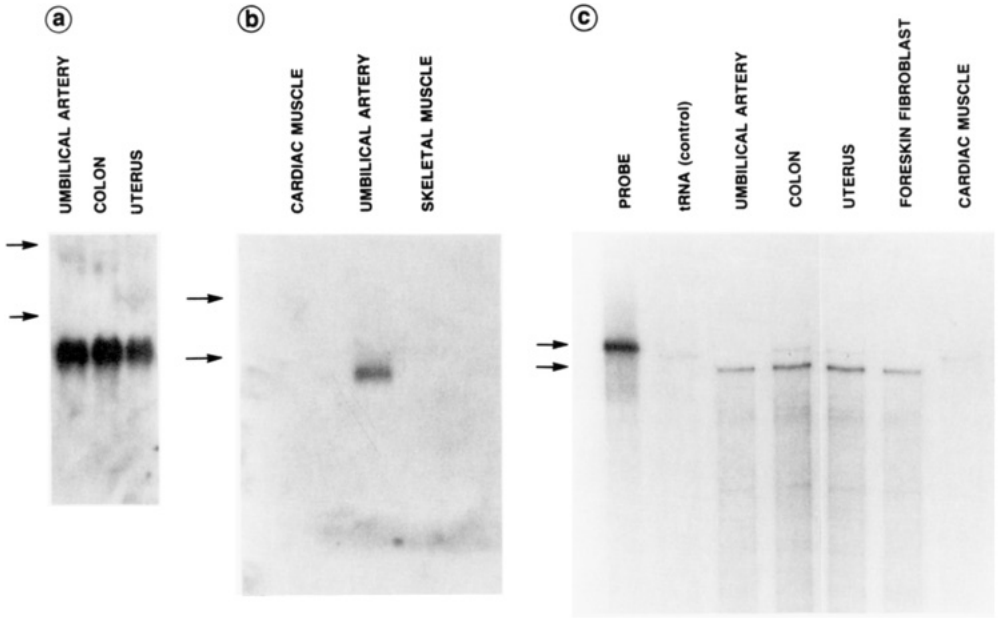

FIGURE 4: Northern blot analysis and S1 nuclease mapping analysis using full-length HuMLC-2 cDNA insert. RNA blot hybridizations using the ^{32}P-labeled MLC-2 cDNA insert obtained from phage λHuMLC-6 were carried out as described under Materials and Methods, using 10 μg of RNA from the following sources: (a) Lane 1, human umbilical artery; lane 2, human colon; lane 3, human uterus tissue; (b) lane 1, human cardiac muscle tissue; lane 2, human umbilical artery; lane 3, human skeletal muscle tissue; the mobilities of the 28S and 18S ribosomal RNAs in (a) and (b) are indicated by arrows. (c) S1 nuclease analysis. A uniformly labeled single-stranded DNA was obtained by elongating the M13 universal primer annealed to the M13 mp9 template carrying a full-length HuMLC-2 cDNA insert in the sense orientation (see Materials and Methods). Total RNA (20 μg) isolated from the muscle tissues indicated above was used for the hybridization to ssDNA probes, and 1000 units of S1 nuclease was used per assay. The top arrow denotes the ssDNA probe used in hybridization analysis, and the bottom arrow denotes the ssDNA protected with S1 nuclease after hybridization to RNA. Electrophoresis of the digested products was done on a thin 6% polyacrylamide–urea gel.

cell lines but not in the hemopoietic cell lines. Similarly, normal unactivated T cells also do not show a hybridizable RNA species (data not shown). Longer exposure of the Northern blot failed to indicate a low-level expression of MLC-2 mRNA in the hemopoietic cells. However, hybridization and washing the filters under conditions of low stringency do light up another RNA species in all the cell lines (Figure 5c). Rehybridization of the same blot with a human β-actin cDNA probe as a positive control (Figure 5b) shows that the β-actin mRNA is readily detectable in all the cell lines and that the quality of the RNA preparation is good. Hence, unlike the rat aortic MLC-2 isoform, which is shown to be constitutively expressed in all muscle and nonmuscle cells, the human smooth MLC-2 isoform is expressed restrictively in only smooth muscle tissues and in some, but not all, nonmuscle cells.

Western blot analysis of cell lysates from the nonmuscle cell lines (fibroblast and hemopoietic) using antibodies raised against chicken gizzard MLC-2 protein showed that the MLC-2-related protein species is readily detectable in all of them (data not shown). Hence, the lack of hybridizing RNA species in these nonmuscle cells is not due to very low levels of MLC-2 protein. These results suggest that certain nonmuscle cells express a different MLC-2 isoform whose mRNA does not cross-hydridize with the MLC-2 cDNA clone characterized in the present study. It should be noted that even though we used the rat aortic MLC-2 cDNA probe to identify the human smooth muscle clone, the conditions for washing the filters were less stringent than the ones used for Northern blot analysis (see Materials and Methods).

To explore the possibility that nonmuscle cell lines might be expressing more than one MLC-2 isoform and to compare the distribution of MLC-2 isoforms in different nonmuscle cells, we undertook two-dimensional protein gel electrophoresis analysis. The ^{35}S-labeled protein extracts of human lung fibroblast cell line WI38 and the immunoprecipitates using MLC-2 antiserum were resolved on two-dimensional gels. As shown in Figure 6a,b, the two-dimensional gel system resolves three protein species that are recognized by antibodies against purified chicken gizzard MLC-2. Recently Kawamoto and Adelstein (1988) have also demonstrated three MLC-2 isoforms in rat aortic smooth muscle cells in culture, based on two-dimensional gel analysis. Together these results point to the existence of three MLC-2 protein isoforms in human nonmuscle cell lines. Comparison of the two-dimensional gel patterns of human T cells that do not express the smooth muscle MLC-2 mRNA with that of the fibroblast cells shows the specific absence of one isoform, and the other two species are present at normal levels (Figure 7). The in vitro synthesized MLC-2 protein corresponding to the cloned cDNA (Figure 2) comigrates on the two-dimensional gel system with the species that is specifically absent in the T cells (unpublished observations). These results indicate that some nonmuscle cell lines express one smooth muscle and two nonmuscle MLC-2 isoforms, whereas others such T cells express only two nonmuscle isoforms.

Discussion

We report here the isolation of cDNA clones encoding the 20-kDa myosin light chain 2 from human vascular smooth muscle tissue. Clones HuMLC-1, -3, and -6 represent near-full-length transcripts of the smooth muscle MLC-2 mRNA containing both 5′- and 3′-untranslated regions. The deduced amino acid sequence of the coding region shows striking homology with that of the chicken gizzard MLC-2 and rat aortic MLC-2 with only 3 and 13 amino acid substitutions respectively, suggesting that the cDNA encodes a smooth muscle MLC-2 isoform. The conservation of primary structure among the chicken gizzard MLC-2, rat aortic MLC-2, and the human vascular smooth muscle MLC-2 is somewhat higher

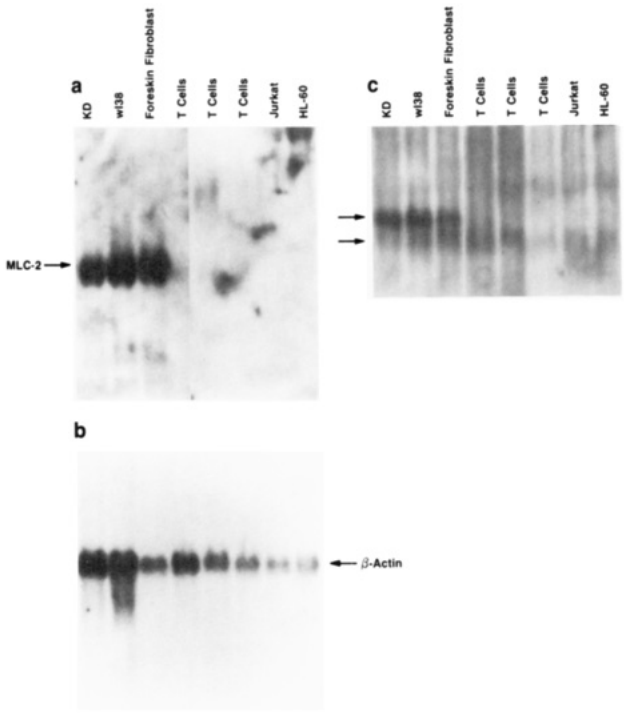

FIGURE 5: (a) MLC-2 mRNA expression in nonmuscle cell lines. Human cell lines were grown to confluence, and total RNA was isolated as described under Materials and Methods. Northern blot hybridization was carried out using 10 μg of total RNA electrophoresed on 1.5% agarose–formaldehyde gels. Hybridization and washing under stringent conditions are as described under Materials and Methods. Final wash was at 0.1× SSC/1% SDS at 65 °C for 30 min. (b) The same RNA blot was rehybridized with a human β-actin cDNA probe (gift from Larry Kedes). (c) The same RNA blot was rehbyridized with the human MLC-2 cDNA probe and washed 3 times at low stringency (2× SSC/0.1% SDS at 37 °C) for 30 min each. The top arrow denotes the RNA species detectable under strong hybridization conditions. The bottom arrow denotes the RNA species detectable under low-stringency hybridization and washing conditions.

than that reported for species differences among the light chains of cardiac and skeletal muscles (Kumar et al., 1986). This may reflect the importance of the primary structure in providing the smooth muscle MLC-2 with its unique properties in regulating both actin-activated myosin ATPase activity and smooth muscle contraction. Kemp et al. (1985) have reported that the sequence at the amino terminus of chicken smooth muscle MLC-2 is important to the activity of the smooth muscle myosin light chain kinase on MLC-2. Hence, it is not surprising that this area is most highly conserved among all the smooth muscle MLC-2s. Despite the differences in the primary structure of different MLC-2s of skeletal, cardiac, and smooth muscle isoforms, there are a number of areas where the sequences are highly conserved. This argues strongly that the gene encoding the putative smooth muscle MLC-2 originated by duplication of the same ancestor that gave rise to multiple MLC-2 genes.

The following features distinguish the human smooth muscle MLC-2 cDNA clone reported in this paper from the previously reported rat aortic MLC-2 cDNA. (1) The human MLC-2 cDNA was isolated from an intact vascular smooth muscle tissue as opposed to the rat aortic MLC-2 cDNA which was isolated from smooth muscle cells in culture (Taubman et al., 1987). It is known that smooth muscle cells in culture express both contractile and noncontractile phenotypes, which are both distinct and reversible (Chamley-Campbell et al., 1979). Recently, Kawamoto and Adelstein (1988) and Csabina et al. (1986) have identified three MLC-2 isoforms in rat aortic smooth muscle cells in culture based on immunoprecipitation followed by two-dimensional gel analysis. It has been demonstrated that an inverse relationship exists between smooth muscle α-actin expression and cellular proliferation in cultured rat aortic smooth muscle cells (Owens et al., 1986). Smooth muscle cells in culture express predominantly nonmuscle contractile isoforms during the proliferative stage, and the muscle-specific isoforms are induced when the cells reach confluence and enter into a contractile state (Larson et al., 1982). (2) The rat aortic MLC-2 cDNA is expressed ubiquitously in all muscle and nonmuscle tissues, and the possibility was raised either that it encodes actually a nonmuscle isoform or that the smooth muscle and nonmuscle isoforms are identical and products of the same gene. Our results differ somewhat from those obtained by Taubmann et al. (1987) in that the human MLC-2 isoform characterized from an intact smooth muscle tissue is definitely not expressed in all muscle and nonmuscle cells. Its expression is restricted to smooth

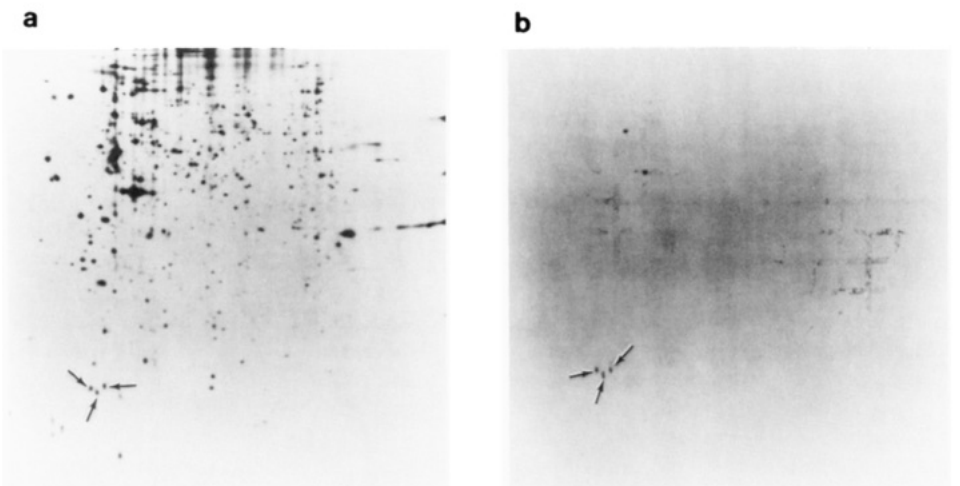

FIGURE 6: MLC-2 isoforms in human lung fibroblast cells. Two-dimensional gel electrophoresis of total [^{35}S]methionine-labeled polypeptides from human lung fibroblast cell line (WI 38) was performed using the QUEST gel lab facility at Cold Spring Harbor Laboratory (a). Labeling with [^{35}S]methionine was for 16 h, and the proteins were resolved with pH 3.5–10 ampholytes in the first dimension and 12.5% acrylamide in the second dimension. The gels were fixed in methanol/acetic acid and processed for fluorography using Enhance (New England Nuclear) as suggested by the manufacturer and exposed to Kodak XAR film. Proteins immunoprecipitated with MLC-2 antibodies were also analyzed on the two-dimensional gel system (b). The three MLC-2 species are indicated by arrows.

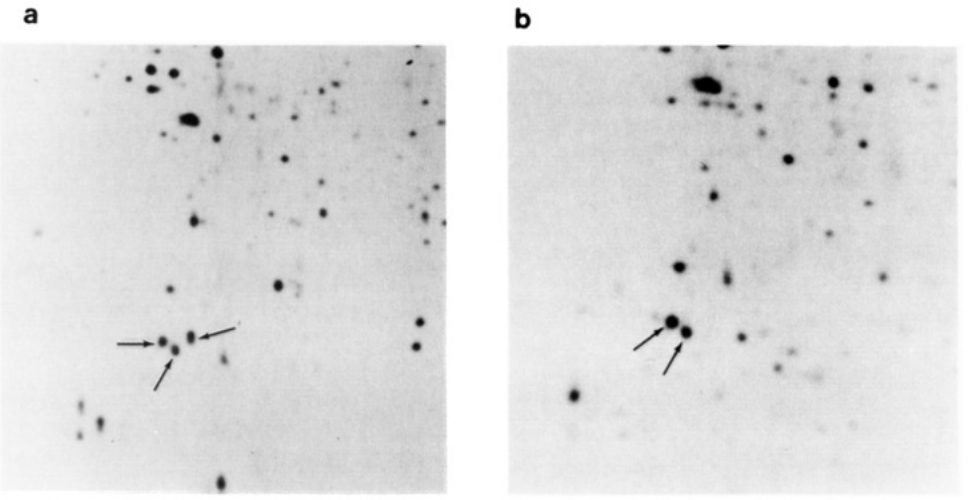

FIGURE 7: Comparison of the MLC-2 isoforms in human lung fibroblast cells and T cells using two-dimensional gel electrophoresis. Proteins of human lung fibroblast cells and T cells were labeled at confluence and were resolved on the two-dimensional gel system, as descried in the legend to Figure 6. The gels were processed for fluorography and exposed to X-ray film for 4 weeks. An enlarged region of the MLC-2 in the fibroblast cell line is compared with the same region in T cells. The MLC-2 isoforms are identified by arrows.

muscle tissues and to some nonmuscle cells of mesenchymal origin. These results imply that there is one other (or perhaps more) nonmuscle isoform that is expressed in all human nonmuscle cells. Analysis of the MLC-2 immunoprecipitates using two-dimensional gel electrophoresis confirms that fibroblast cell lines express one smooth muscle specific and two nonmuscle MLC-2 isoforms whereas the hemopoietic cells, such as T cells, express only the two nonmuscle isoforms. Hence, we believe that the rat aortic MLC-2 cDNA corresponds to a nonmuscle isoform since it is expressed in all cells constitutively and the cDNA characterized in this report corresponds to a novel distinct smooth muscle isoform. (3) In view of this, it is interesting to note that there are a number of amino acid substitutions in the rat aortic MLC-2 isoform compared to the chicken gizzard and human smooth muscle MLC-2 isoform. These substitutions are located between amino acids 69 and 74 and also between 113 and 121 (Figure 2). In other words, the human MLC-2 isoform that we have characterized shows slightly greater homology to the published chicken gizzard protein sequence than the rat aortic MLC-2 isoform. In fact, we have recently characterized two highly homologous but distinct MLC-2 cDNA clones from chicken gizzard tissue, one of which, like the rat MLC-2 clone, is expressed in all muscle and nonmuscle tissues, and another one, like the human clone described in this paper, is expressed restrictively in smooth muscle tissues (Zavodny et al., 1988; unpublished results). The sequence divergence between the two chicken MLC-2 cDNAs is random and distributed throughout the coding and noncoding regions, precluding the possibility of a single gene giving rise to two highly homologous isoforms by differential splicing. These results indicate that the smooth muscle and nonmuscle isoforms are distinct and are encoded by separate genes.

Smooth muscle cells play a key role in the progression of atherosclerosis (Schwartz et al., 1986). Following an initiating event, migration of smooth muscle cells from the medial layer to the intima triggers abnormal smooth muscle cell proliferation. Evidence indicates that the smooth muscle cell population within the arterial lesion can be of monoclonal origin (Benditt & Benditt, 1973; Benditt, 1977). Changes in the expression of contractile protein genes are seen not only in cell culture but also in areas of atherosclerotic plaque and blood vessel injury (Gabbiani et al., 1984; Owens et al., 1986). Specifically, rat aortic smooth muscle cells in culture and the human atheromatous plaque cells develop a switch in actin isoform expression from a predominance of the α-smooth muscle type to a predominance of the γ-nonmuscle type (Gabbiani et al., 1984). Cultured smooth muscle cells also show changes in myosin expression according to the culture conditions (Larson et al., 1982). Our preliminary results indicate that the smooth muscle MLC-2 isoform is repressed when cells undergo transformation (unpublished observation). The availability of a smooth muscle MLC-2 probe should be useful to study the mechanisms involved in the regulation of this isoform during transformation and also possibly in vivo during the development of atherosclerotic plaques.

ACKNOWLEDGMENTS

We are grateful to Drs. Mark Taubman and B. Nadal-Ginard for providing us with rat aortic MLC-2 cDNA. We thank Dr. Marian Plunkett for help with the cell culturing and Herinder Lonial, Steve Dailey, and Mary Petro for their expert technical help. We are grateful to Dr. Jim Garrels (Cold Spring Harbor) for the use of the QUEST 2D-gel lab facility. We thank Dr. Earl Ruley for his helpful comments on the manuscript and Dr. Claude Nash for his encouragement and support throughout the course of this investigation.

Registry No. MLC-2, 119793-80-5; MLC-2 cDNA, 119793-79-2.

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