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Dictyostelium discoideum Myosin: Isolation and Characterization of cDNAs Encoding the Regulatory Light Chain

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Phosphorylation of the regulatory light chains (RMLC) of nonmuscle myosin can increase the actin-activated ATPase activity and filament formation. Little is known about these regulatory mechanisms and how the RMLC are involved in ATP hydrolysis. To better characterize the nonmuscle RMLC, we isolated cDNAs encoding the *Dictyostelium* RMLC. Using an antibody specific for the RMLC, we screened a λgt11 expression library and obtained a 200-base-pair clone that encoded a portion of the RMLC. The remainder of the sequence was obtained from two clones identified by DNA hybridization, using the 200-base-pair cDNA. The composite RMLC cDNA was 645 nucleotides long. It contained 60 base pairs of 5′ untranslated, 483 bases of coding, and 102 base pairs of 3′ untranslated sequence. The amino acid sequence predicted an 18,300-dalton protein that shares 42% amino acid identity with *Dictyostelium* calmodulin and 30% identity with the chicken skeletal myosin RMLC. This sequence contained three regions that were similar to the E-F hand calcium-binding domains found in calmodulin, troponin C, and other myosin light chains. A sequence similar to the phosphorylation sequence found in chicken gizzard and skeletal myosin light chains was found at the amino terminus. Genomic Southern blot analysis suggested that the *Dictyostelium* genome contains a single gene encoding the RMLC. Analysis of RMLC expression patterns during *Dictyostelium* development indicated that accumulation of this mRNA increases just before aggregation and again during culmination. This pattern is similar to that obtained for the *Dictyostelium* essential myosin light chain and suggests that expression of the two light chains is coordinated during development.

In nonmuscle cells, the actomyosin-based contractile system has been shown to be involved in many cellular functions, such as intracellular transport (52), cytokinesis (19, 45, 48, 54, 55), phagocytosis (60), receptor capping (26), endocytosis and exocytosis (3), and pseudopodial and filipodial extension (2, 56). However, although the actomyosin contractile system is required for a number of nonmuscle cell phenomena, the functional structures and regulatory mechanisms of these systems are not well understood. Unlike muscle cell contractile systems, nonmuscle systems do not maintain a strict molecular organization such as that found in the sarcomere. The proteins involved are required for a multitude of phenomena and are reorganized accordingly. Using indirect immunofluorescence, Carboni and Condeelis (6) have demonstrated the rearrangement of actin, myosin, α-actinin, and the 95-kilodalton (kDa) protein during concavalin A-induced capping in *Dictyostelium discoideum*. Furthermore, using immunofluorescence, Yumura et al. (70) have demonstrated that myosin is present within the contractile ring of dividing *Dictyostelium* amoeba and in the cellular cortex of actively moving cells. Because of the dynamic nature of each component, elucidation of the structure and regulation of the individual components may aid in our understanding of the entire contractile mechanism.

Myosin monomers isolated from *D. discoideum* contain two 240,000-Da heavy chains (MHC), two 18,000-Da regulatory light chains (RMLC), and two 16,000-Da essential light chains (EMLC). The catalytic and actin-binding domains are localized to the globular head region of the MHC. Sequencing data reveal that these domains are highly homologous to the ATPase and actin-binding domains of muscle myosin (64). The tail region of the MHC is required for assembly of the bipolar thick filament. Although this region is not homologous to similar regions in muscle myosin, the *Dictyostelium* MHC does contain amino acid patterns required for formation of the alpha-helical-coiled coil. In addition, the *Dictyostelium* MHC contains a 196-amino-acid repeat of charged amino acids that is believed to participate in filament formation. Phosphorylation of threonine residues within this repeat (61) decreases both the filament assembly and actin-activated Mg^{2+}-ATPase activity of *Dictyostelium* myosin (33).

The RMLC are associated with the head region of the MHC. The EMLC is believed to function in the formation of the ATPase active site (41) and actin binding (46). As determined by cDNA sequencing, the *Dictyostelium* EMLC displays 30% amino acid sequence identity with the chicken skeletal muscle EMLC and contains three regions homologous to the consensus E-F hand calcium-binding domains common to most RMLC (8). The RMLC has been shown to modulate the enzymatic properties of myosin (1, 21, 25, 43, 53, 58). In smooth muscle and many nonmuscle systems, the Ca^{2+}-dependent phosphorylation of the RMLC by myosin light-chain kinase increases the actin-activated ATPase activity of the myosin (1, 20, 53, 58). RMLC phosphorylation has been implicated in the regulation of platelet aggregation (13, 14), capping in lymphocytes (5), and smooth muscle tension (35). In *D. discoideum*, cyclic AMP (cAMP) stimulation induces RMLC phosphorylation (4); however, little is known about how phosphorylation affects myosin function in vivo.

The single-celled amoeba *D. discoideum* is an excellent system for the study of the regulation and function of nonmuscle cell myosins. *Dictyostelium* amoeba can be grown in large quantities, which has allowed the purification and characterization of many of the nonmuscle contractile

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components, including actin (59), myosin I (11), myosin II (9), and the myosin kinases (20, 32, 38). In addition, throughout its life cycle, this organism exhibits several types of cell motility. This characteristic has enabled the analysis of events involved in motility, such as the regulation and organization of myosin within the cell. For example, using indirect immunofluorescence, Yumura and Fukui (69) have demonstrated that myosin is preferentially localized in the posterior cortex of the chemotaxing cell and that in the developmentally competent amoeba, myosin transiently relocalizes in response to cAMP stimulation. Berlot et al. (4) have been able to correlate this transient relocation of myosin with increases of heavy- and light-chain phosphorylation that are induced during cAMP stimulation.

Finally, application of molecular genetic techniques to *D. discoideum* has made it possible to disrupt expression and modify gene structure in vivo (15, 29, 40, 67). Disruption or modification of the MHC indicates that a functional MHC is required for cytokinesis and development (30), yet is not essential for cell locomotion or directed cell movement (29, 44, 66). Similar molecular experiments will provide a powerful approach for studying the function of the RMLC in vivo.

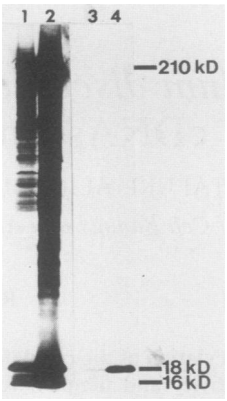

FIG. 1. Identification of the epitope encoded by the λgt11 cDNA clone RMLC-1. Antibody specific for *Dictyostelium* myosin (NU3) was affinity purified by the epitope selection procedure, using the RMLC-1 cDNA clone. Both the purified antibody (lanes 3 and 4) and the original antiserum (lanes 1 and 2) were used to stain a whole-cell protein lysate (lanes 1 and 3) and purified myosin (lanes 2 and 4). The epitope-selected antibody recognized only the RMLC in both purified myosin and a whole-cell protein lysate.

MATERIALS AND METHODS

Isolation and screening of cDNA clones. The λgt11 cDNA library used to isolate the MLC-R1A and RMLC-51 clones was derived from mRNA obtained from *Dictyostelium* cells developed for 12 h in the presence of cAMP (8). RMLC-k1 was obtained from a λgt11 cDNA library constructed from 4-h developed cell mRNA (generously provided by Richard Kessin and his colleagues, Columbia University, New York, N.Y.). Antibody screening as previously described (8) was used to isolate RMLC-1. Epitope selection was used to confirm the identity of RMLC-1 (65). To obtain the additional clones, plaque lifts of each library were probed with ^{32}P-labeled DNA probes (17, 18). RMLC-2 was identified by using the ^{32}P-labeled RMLC-1A insert, whereas RMLC-k1 was identified by a ^{32}P-labeled restriction fragment of RMLC-2.

Subcloning and sequencing. Subcloning of the *Eco*RI inserts from RMLC-1, RMLC-2, and RMLC-k1 was done by standard methods (37). The resulting clones were sequenced on single- and double-stranded templates by chain termination sequencing techniques (51). DNA sequences of both strands were determined. DNA sequences were analyzed by using the Pustell/IBI (47) and University of Wisconsin Genetic Computer Group (16) software packages and the programs available through the Bionet National Resource.

Molecular hybridization techniques. For Southern blot analysis, restriction enzyme-digested *Dictyostelium* DNA was separated on agarose gels, depurinated in 0.25 M HCl, and transferred to GeneScreen Plus (Dupont, NEN Research Products) in 0.4 M NaOH (49). Filters were prehybridized for at least 3 h at 65°C in DNA-DNA hybridization solution (5× Denhardt solution, 6× SSC buffer [SSC is 0.15 NaCl plus 0.015 sodium citrate], 0.5% sodium dodecyl sulfate [SDS], 20 mM $NaPO_4$ [pH 6.5], 10 μg of calf thymus DNA per ml). After prehybridization, the solution was replaced with fresh hybridization buffer containing 10^6 cpm of labeled probe per ml. Hybridization mixtures were incubated for 12 to 48 h at 65°C. Probes were radiolabeled by random-primed synthesis (17, 18). After hybridization, filters were washed four times for 30 min in 250 ml of 2× SSC buffer–0.1% SDS at 65°C. Filters were exposed to Kodak X-Omat AR film (Eastman Kodak Co.) in the presence of Cronex Lightning-Plus intensifying screens (E. I. du Pont de Nemours & Co., Inc.).

For Northern (RNA) blots, RNA was transferred to nitrocellulose in 10× SSC buffer and baked at 80°C for 2 h under vacuum. Hybridizations were performed in 50% deionized formamide–5× SSC–50 mM $NaPO_4$ (pH 6.5)–0.1% SDS–5× Denhardt solution–10 μg of calf thymus DNA per ml at 37°C. Washes were performed in 0.1× SSC–0.1% SDS at 50°C.

RESULTS

Isolation of RMLC cDNAs. The λgt11 library containing cDNAs representing poly(A)$^+$ RNA isolated from 12-h starved amoeba was screened with a 1:100 dilution of the anti-RMLC antibody NU3 (8). Positive plaques were picked and rescreened until plates contained only plaques reactive to NU3; 25 clones were isolated. Although NU3 reacted most strongly with the myosin light chains, some reactivity to the MHC was observed. To eliminate clones containing MHC sequences, the antibody-positive plaques were rescreened with NU3 that had been preabsorbed against isolated heavy chains (8). Four plaques reacted with the absorbed antibody and presumably contained myosin light-chain sequences. Epitope selection was used to distinguish between RMLC and EMLC clones (8, 65). The preabsorbed NU3 antibody was affinity purified against the fusion proteins produced by the individual clones. The resulting antibodies were reacted to nitrocellulose blots of purified *Dictyostelium* myosin. Antibody affinity purified against the proteins present in the RMLC-1 plaques reacted strongly with the 18-kDa light chain of purified myosin (Fig. 1, lane 4) as well as a single 18-kDa band in a whole-cell lysate (lane 3). Slight cross-reactivity against the 16-kDa light chain was also observed. Perhaps this cross-reactivity resulted from regions of structural similarity between the essential and regulatory light chains.

To examine the nature of the NU3-reactive protein being produced by bacteria infected with λgt11 containing the RMLC-1 cDNA, *Escherichia coli* Y1089 (68) was lysogenized by bacteriophage carrying the RMLC-1 cDNA. After temperature induction of the phage and isopropyl-β-D-thio-

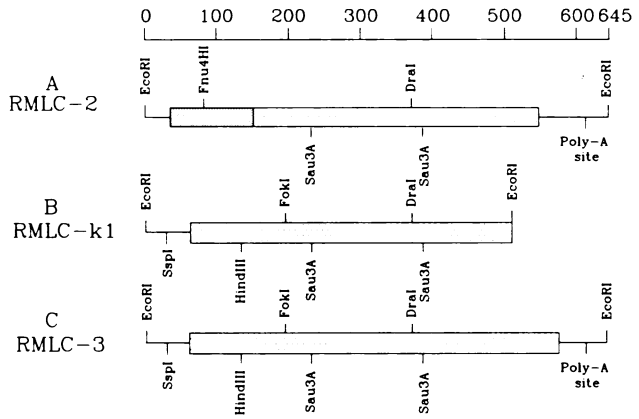

FIG. 2. Partial restriction map of RMLC cDNA clones. Shown are cDNA inserts isolated from RMLC-2 (A), RMLC-k1 (B), and RMLC-3 (C). Lines represent untranslated regions; boxed regions represent coding sequences. The densely stippled area in the RMLC-2 sequence represents the position at which there is a shift in the reading frame.

galactopyranoside (IPTG) induction of β-galactosidase, cells were harvested, suspended in SDS sample buffer, and analyzed by SDS-polyacrylamide gel electrophoresis. β-Galactosidase was absent from RMLC-1 cell extracts; however, a larger protein that was not present in cells infected with unmodified λgt11 was identified. Immunoblotting showed that the new protein reacted with the NU3 antibody, confirming that the RMLC-1 phage was indeed expressing a β-galactosidase–RMLC fusion protein.

*Eco*RI restriction digestion of purified RMLC-1 DNA revealed the presence of two *Eco*RI fragments with lengths of 140 (RMLC-1A) and 194 (RMLC-1B) nucleotides. This finding suggested that either an endogenous *Eco*RI site within the cDNA was protected during the digests required for construction of the library or that two unrelated fragments were ligated to form a single clone. Double-stranded sequencing of the phage DNA showed that RMLC-1A was linked in frame to the β-galactosidase gene, suggesting that this fragment contained RMLC sequences. To provide additional evidence supporting this idea, ^{32}P-labeled probes were made from each fragment and used to probe a blot of RNA isolated from vegetative cells. Only RMLC-1A reacted with an mRNA of a size required to synthesize the 18-kDa RMLC. The RMLC-1B fragment reacted with an mRNA of only about 400 nucleotides, far too short to encode an 18-kDa polypeptide. Because the two fragments reacted with different mRNAs, the two *Eco*RI inserts most likely represented unrelated cDNAs. On the basis of these results, we concluded that the 1A fragment represented an RMLC cDNA.

To obtain a cDNA containing the entire coding sequence for the RMLC, the RMLC-1A fragment was used to rescreen the λgt11 cDNA library. Several positive clones were isolated. A restriction digest with *Eco*RI showed that the clone designated RMLC-2 contained a 643-base-pair insert. The insert from RMLC-2 was subcloned into M13 and Bluescript vectors (Stratagene) and sequenced. Figure 2A shows a partial restriction map of the RMLC-2 cDNA. Although the sequence obtained appeared to contain a typical *Dictyostelium* translation start sequence and methionine, it did not contain a single open reading frame. One reading frame initiated at the suspected start site and continued for 150 bases. The second open reading frame began at base 90 and terminated at the termination codon found at nucleotide 544. The sequence homologous to RMLC-1A resided within the second open reading frame. Comparison of the predicted amino acid sequence with known RMLC protein sequences demonstrated that the sequence encoded by the 3′ portion of the mRNA was similar to that of chicken skeletal RMLC. The 5′ 150 bases showed no homology to any known myosin sequence. To determine whether the 5′ portion of sequence was related to the RMLC, a probe containing the 5′ region of the RMLC was synthesized by a primer extension reaction (37) and hybridized to a blot of restriction enzyme-cut RMLC-2. The primer used for the extension was a synthetic oligonucleotide complementary to the region surrounding the *Fok*I site of RMLC-2. This probe failed to react with the first 100 bases of RMLC-2, suggesting that this sequence did not code for the RMLC. Presumably, this 5′ sequence represents the ligation of two individual cDNAs before addition of linker during construction of the library.

To obtain a clone containing the authentic 5′ end of the RMLC cDNA, the 140-base-pair internal *Sau*3A restriction fragment from the 3′ coding region of RMLC-2 was labeled and used to probe a λgt11 library containing cDNAs representing mRNA isolated from *Dictyostelium* cells developed for 4 h (36). A clone, RMLC-k1, containing a 510-base-pair *Eco*RI insert was isolated. A partial restriction map of RMLC-k1 is presented in Fig. 2B. RMLC-k1 contained an A+T-rich 5′ untranslated sequence, a typical *Dictyostelium* translation start sequence, initiator methionine, and 94% of the RMLC-coding sequence. RMLC-k1 did not contain the last 135 nucleotides of 3′ sequence; however, the RMLC-k1 sequence from bases 150 to 510 was identical to the same regions in RMLC-2. As suspected, the 5′ 150 base pairs was not homologous to the corresponding region of RMLC-2. A complete cDNA was constructed by using the appropriate *Fok*I-*Eco*RI restriction fragments from RMLC-2 and RMLC-k1 and was designated RMLC-3. A partial restriction map of RMLC-3 is presented in Fig. 2C. Figure 3 shows the complete DNA sequence for RMLC and the predicted amino acid sequence of the encoded polypeptide. The sequence contains a 60-nucleotide untranslated leader sequence, a typical A-rich translational start sequence, and initiator methionine. The open reading frame encodes 161 amino acids which constitute a polypeptide with a predicted molecular weight of 18,303. The predicted isoelectric point for this peptide is 5.9, consistent with that observed for the *Dictyostelium* RMLC polypeptides. In addition, the cDNA contains about 100 3′ untranslated nucleotides which contain a eukaryotic consensus polyadenylation sequence (AATAAA) 75 nucleotides downstream from the translation termination signal.

The *Dictyostelium* RMLC displayed amino acid homology with calmodulin, troponin C, and other regulatory light chains. The results of a comparison of the amino acid sequences of *Dictyostelium* calmodulin and the RMLC are shown in Fig. 4. The RMLC showed 42% sequence identity to *Dictyostelium* calmodulin. The RMLC calcium-binding domains corresponded to domains 1 to 3 in the calmodulin sequence. Significant sequence homology was also evident outside the binding domains, particularly in the region preceding domain 1. Figure 5 shows dot matrix sequence homology comparisons between the *Dictyostelium* RMLC and the chicken cardiac 2b, rabbit skeletal muscle, and smooth muscle RMLC. Sequence homology was spread evenly throughout the peptides and included the regions containing calcium-binding and phosphorylation sites. The

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        10                  30                  50
GGAATTCGGTCAAATTTCTTCAATTCTTTGTATTAAATATTACTCAAACATAAAATAAAA

        70                  90                  110
ATGGCCTCAACCAAAAGAAGATTAAACAGAGAAGAATCATCTGTAGTTTTAGGTGAAGAA
MetAlaSerThrLysArgArgLeuAsnArgGluGluSerSerValValLeuGlyGluGlu

        130                 150                 170
CAAGTTGCTGAATTAAAAGAAGCTTTTGAACTCTTTGATAAAGATAGAACTGGTTTCATT
GlnValAlaGluLeuLysGluAlaPheGluLeuPheAspLysAspArgThrGlyPheIle

        190                 210                 230
AAAAAGGATGCCTTAAAAACCACCTGTAAACAATTTGGTGTTTTTGTTATGGAAGATCAA
LysLysAspAlaLeuLysThrThrCysLysGlnPheGlyValPheValMetGluAspGln

        250                 270                 290
TTAGATGCCATGTTTGCTGAAGCTGATACCACCAAATCTGGTGCTATTGGTTTCCCAGAA
LeuAspAlaMetPheAlaGluAlaAspThrThrLysSerGlyAlaIleGlyPheProGlu

        310                 330                 350
TTTATGTCAATGATGTCCCGTCGTATGAAACAAACTTCAAATGAACAAATTTTAATGAAC
PheMetSerMetMetSerArgArgMetLysGlnThrSerAsnGluGlnIleLeuMetAsn

        370                 390                 410
GCTTTTAAAACTTTTGATCCAGAAGGTAATGGTTACATCTTAACAAAAGATTTATCTAAA
AlaPheLysThrPheAspProGluGlyAsnGlyTyrIleLeuThrLysAspLeuSerLys

        430                 450                 470
GCCTTAACAACTTTGGGTGATAAATTAACTGAAGCAGAGTTACAAGAATTGTTATCAATT
AlaLeuThrThrLeuGlyAspLysLeuThrGluAlaGluLeuGlnGluLeuLeuSerIle

        490                 510                 530
TCAGAAAACGAACAAAAGCAAGTTAAATATGACCTTTTCGTTAATACTCTCTTCAGTAAA
SerGluAsnGluGlnLysGlnValLysTyrAspLeuPheValAsnThrLeuPheSerLys

        550                 570                 590
AAATAAAGTGATTATTAAATAGTGGAAAAAAAAAAAAAAAGTATGTTAAAAACGTTTAGGG
Lys

        610                 630
AGAAGAAATTAAATAAAAAAAAAAAAAAAATATATAAAAAAAACAA
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FIG. 3. Nucleotide and amino acid sequences of RMLC-3 cDNA. The predicted amino acid sequence is depicted below the corresponding nucleotide codons.

most divergent portions were located at the carboxyl terminus.

Southern blot analysis of the RMLC gene(s). To determine the number of RMLC genes in the *Dictyostelium* genome, the RMLC-3 cDNA was labeled and used to probe *Dictyostelium* DNA that had been digested with variety of restriction enzymes (Fig. 6). A single restriction fragment containing the RMLC was detected in several digests produced by enzymes that do not cut within the cDNA. This finding suggests that there is a single copy of the RMLC gene within the genome. Further restriction analysis has indicated that the gene contains at least two introns (data not shown). We are currently isolating genomic clones to determine the organization of the RMLC gene.

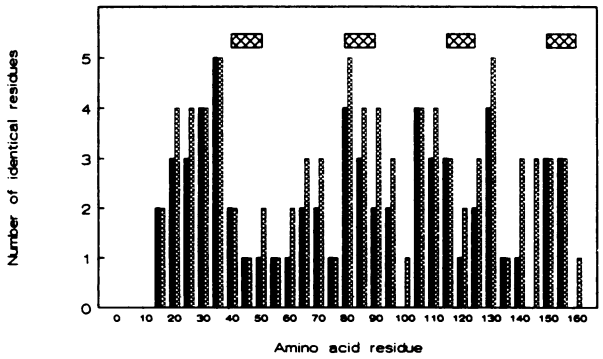

FIG. 4. Histogram representing the sequence conservation between *Dictyostelium* calmodulin and the *Dictyostelium* RMLC. Each bar represents the number of identical residues present within a five-amino-acid window. Filled bars represent absolute sequence homology; sequence identities (hatched bars) include conservative amino acid changes. Hatched boxes at the top indicate the positions of the Ca^{2+}-binding domains in *Dictyostelium* calmodulin.

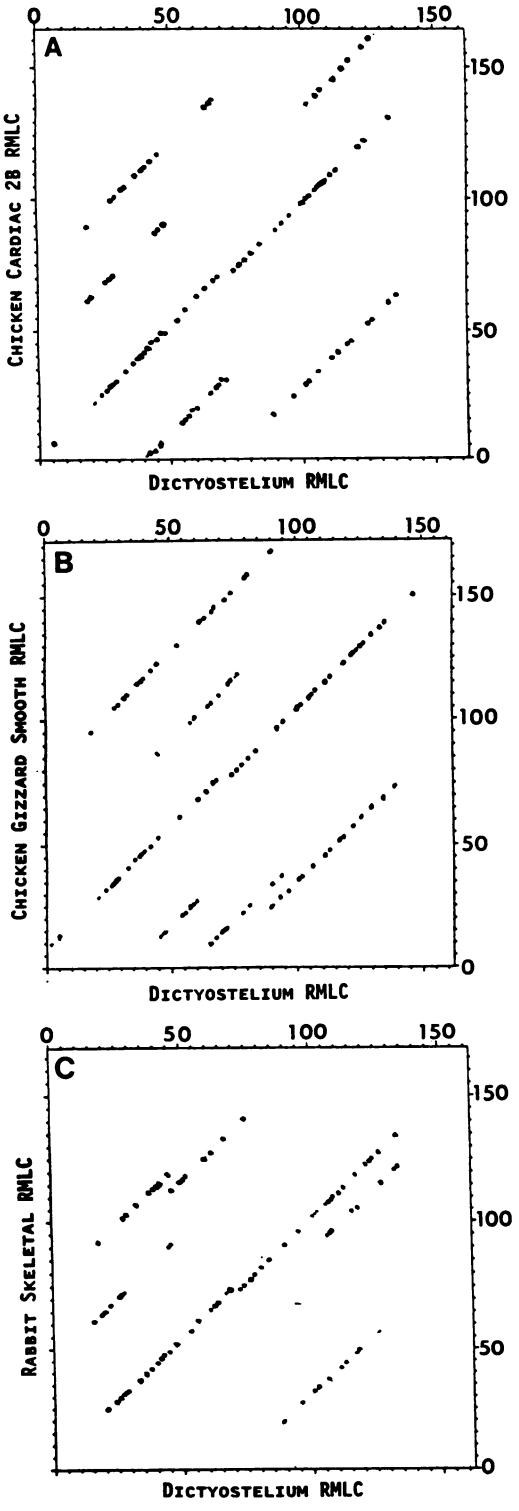

FIG. 5. Dot matrix plot showing the extents and locations of homologous regions between the amino acid sequences of the *Dictyostelium* RMLC and the chicken cardiac 2b (A), chicken gizzard smooth muscle (B), and rabbit skeletal (C) RMLC. Each dot represents identity of at least 8 of 30 amino acids.

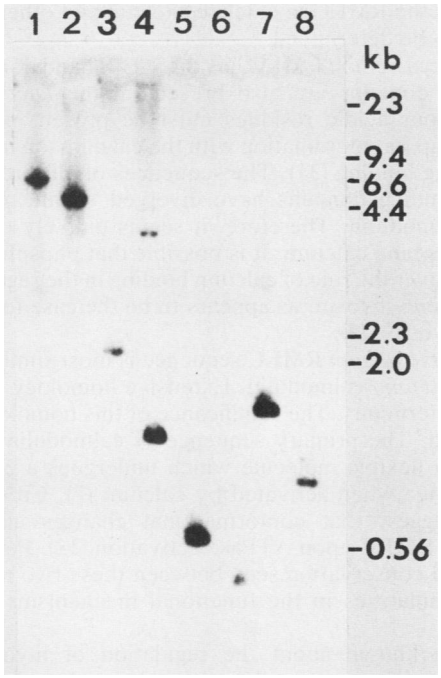

FIG. 6. Southern blot analysis of *Dictyostelium* genomic DNA. Autoradiograph of restriction enzyme-cut genomic *Dictyostelium* DNA that has been probed with ^{32}P-labeled RMLC-3 cDNA. The single bands seen in several of the digests indicate the presence of a single RMLC gene within the *Dictyostelium* genome. Lanes: 1, *Cla*I; 2, *Eco*RI; 3, *Fok*I; 4, *Hin*dIII; 5, *Hin*fI; 6, *Hph*I; 7, *Rsa*I; 8, *Ssp*I. kb, Kilobases.

Developmental regulation of the RMLC mRNA. The rate of cell movement in *D. discoideum* increases during the aggregative stages of development (62). To determine whether the increases in cell motility are associated with increased levels of RMLC mRNA, we examined the expression of RMLC mRNA throughout development by Northern blot analysis. Total cellular RNA was prepared from cells harvested at 4-h intervals during development. Equal amounts of RNA were loaded in each lane of the gel. Equivalent RNA loading was confirmed by ethidium bromide staining and comparison of the amounts of 27S and 17S rRNAs. Blots were probed with the cDNA isolated from RMLC-3. A single 800-base-pair mRNA was detected (Fig. 7A). The size of the detected mRNA was sufficient to encode a polypeptide of 18 kDa and was similar to that seen in other systems. Densitometry was used to determine the level of mRNA present within each sample. After 4 h of development, the levels RMLC mRNA were fivefold higher than in vegetative cells (Fig. 7B), suggesting that aggregation may indeed be associated with increases of RMLC synthesis. At 8 and 12 h, levels of RMLC mRNA returned to values similar to those seen in vegetative cells. A 2.6-fold accumulation occurred at 16 and 20 h. This period corresponds to the point in development when the stalk and fruiting body are forming. This pattern of expression was similar to that we previously reported for the EMLC (8).

DISCUSSION

In this report, we describe the isolation and characterization of cDNAs encoding the RMLC from *D. discoideum*. The RMLC-3 cDNA encoding the RMLC is 645 nucleotides

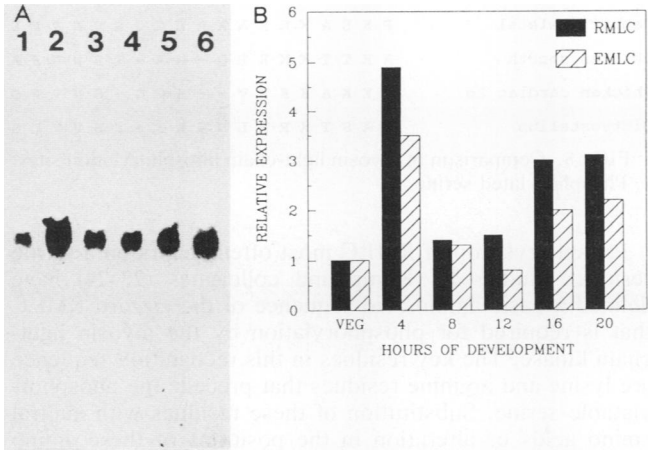

FIG. 7. Developmental regulation of the RMLC mRNA. (A) *Dictyostelium* total RNA was isolated from cells at different stages during development and probed with ^{32}P-labeled RMLC-2. Lanes: 1, vegetative cell RNA; 2 through 6, RNA isolated from cells after 4 (lane 2), 8 (lane 3), 12 (lane 4), 16 (lane 5), and 20 (lane 6) h of development. bp, Base pairs. (B) Histogram showing levels of RMLC (■) and EMLC (▨) mRNAs. Densitometry of the autoradiograph indicates that synthesis of RMLC-2 mRNA increased fivefold after 4 h of development, returned to vegetative levels by 8 h, and again increased slightly after 12 h.

long and contains a single open reading frame initiating at a methionine codon positioned at nucleotide 60. Similar to other *Dictyostelium* mRNAs, including that for the EMLC (8), and MHC (64), and actin (28) genes, the 5′ untranslated sequence is A+T rich and the RMLC translation initiation codon is preceded by an A stretch. A consensus polyadenylation site at nucleotide 610 follows the TAA translation termination codon generally found in *D. discoideum* (27). The cDNA encodes a protein of 161 amino acids with a predicted molecular mass of 18,300 Da. This value agrees with the molecular mass determined by SDS-polyacrylamide gel electrophoresis (9). The calculated pI for this protein of 5.9 is in agreement with the values obtained from isoelectric focusing studies (unpublished results).

RMLC cDNAs have been cloned from skeletal, smooth, and cardiac muscle cells from a variety of species. Sequence analysis indicates that the RMLC from a given muscle type are highly homologous between species, whereas those from different muscle systems within the same organism show much less homology. For example, there is 83% homology between rat and chicken cardiac RMLC versus 67% homology between rat cardiac and skeletal RMLC (34). Perhaps these sequence variations are responsible for specific functional differences. The *Dictyostelium* RMLC amino acid sequence is 42% homologous to *Dictyostelium* calmodulin, 34% homologous to the chicken cardiac 2b RMLC, 32% homologous to the smooth muscle RMLC, and 30% homologous to the rabbit skeletal RMLC. The sequence identity among the RMLC is spread evenly throughout the peptides and includes the potential calcium-binding domains and the phosphorylation sites. Presumably, a portion of the remaining regions is responsible for MHC binding. The sequences diverge significantly in the carboxy-terminal portions of the peptides. Moreover, the amino acid sequence of the *Dictyostelium* EMLC carboxyl terminus also diverges from that of other EMLC. It is not known whether these sequence variations define functional differences between muscle and nonmuscle RMLC.

Rabbit skeletal	P K K A K R R A A A E G G S S N V F S	*
Chicken smooth	A K T T K K R P Q - R A - T S N V F A	*
Chicken cardiac 2b	P K K A K K K V - - E G G - S N V F S	*
Dictyostelium	M A S T K R R L N R E E - S S V V L G	?

FIG. 8. Comparison of myosin light-chain phosphorylation sites. *, Phosphorylated serine.

Phosphorylation of RMLC most often occurs on a serine residue. Studies by Kemp and colleagues (22–24) have defined a basic amino acid sequence of the gizzard RMLC that is required for phosphorylation by the myosin light-chain kinase. The key residues in this recognition sequence are lysine and arginine residues that precede the phosphorylatable serine. Substitution of these residues with neutral amino acids or alteration in the positions of these amino acids disrupts or alters phosphorylation. In addition, the four amino acids on the carboxyl-terminal side of the phosphorylation site have a strong influence on the reaction kinetics (42). Removal of the valine or phenyalanine residues in this sequence decreases the V_{max} of the phosphorylation reaction. Sequence comparisons have revealed that all phosphorylatable RMLC contain similar amino acid domains. Similar basic amino acid sequences are used as phosphorylation recognition sequences by a variety of cellular kinases (22).

The *Dictyostelium* RMLC is phosphorylated on a serine residue (4). Preceding serine-13 and serine-14 in the *Dictyostelium* RMLC are several arginine and lysine residues. These residues are positioned similarly to those in the gizzard RMLC (Fig. 8). However, the carboxyl-terminal sequence following the putative phospho residues diverges significantly from that seen in other RMLC. This is unusual, since this sequence is absolutely conserved in all reported muscle RMLC sequences. Studies have indicated that the *Dictyostelium* myosin light-chain kinase is highly specific and will not phosphorylate myosins isolated from rabbit skeletal and cardiac muscle, turkey smooth muscle, platelet, or *Acanthamoeba* cells. Moreover, gizzard myosin light-chain kinase does not phosphorylate the *Dictyostelium* RMLC (20; S. R. Tafuri and E. R. Kuczmarski, J. Cell Biol. **101**:160a, 1985). The variations seen in sequence surrounding the phosphorylation site may explain the specificities of these enzymes. Further analysis is required to determine which serine is the phosphorylated residue.

The Ca^{2+}-binding regions of the RMLC demonstrate the highly conserved E-F hand structure common to many calcium-binding proteins, including calmodulin and troponin C (31). This structure is found in the essential and regulatory light chains of most myosins studied (10). In several systems, such as scallop and chicken skeletal muscle, evidence suggests that at least one of these domains is functional. The binding of Ca^{2+} to the head region of scallop myosin is responsible for the stimulation of the actin-activated myosin ATPase activity (25). The scallop RMLC is not phosphorylated. Loss of RMLC from scallop myosin results in desensitization to calcium regulation. RMLC from other sources can be reassociated the densensitized myosin and tested to see whether they can restore calcium sensitivity to scallop myosin. The chicken skeletal RMLC prevents calcium activation of scallop myosin activity. However, mutation of calcium-binding domain I of the chicken skeletal RMLC relieves this inhibition (50). This finding suggests that the chicken RMLC does actively bind calcium and that this binding regulates the interaction between actin and myosin in scallop hybrids. At present, the function of calcium binding by the RMLC in chicken skeletal and other systems remains to be determined.

The *Dictyostelium* EMLC has three E-F hand domains (8), and three domains are also present in the *Dictyostelium* RMLC. Four acidic residues must be present within the binding loop for coordination with the calcium ion in order to have strong binding (31). The sequences of all three RMLC calcium-binding domains have diverged significantly from that of calmodulin. Therefore, it seems unlikely that these light chains bind calcium. It is possible that phosphorylation has taken over the role of calcium binding in the regulation of *Dictyostelium* myosin, as appears to be the case for smooth muscle myosin (25).

The *Dictyostelium* RMLC sequence is most similar to that of *Dictyostelium* calmodulin. Extensive homology occurs at the amino terminus. The significance of this homology is not understood. The primary sequence of calmodulin suggests that it is a flexible molecule which undergoes a conformational change when activated by calcium (7). Cross-linking studies suggest that conformational changes also occur within the RMLC upon ATPase activation (12). Perhaps the amino acid conservation seen between these two molecules reflects similarities in the functional mechanisms of these proteins.

Little is known about the regulation of myosin gene expression. Questions still to be answered include those concerning how myosin subunit expression is coordinated, how myosin expression is synchronized with that of other contractile proteins, and which cellular events require myosin synthesis. During *Dictyostelium* development, synthesis of RMLC mRNAs increases just before aggregation and again a few hours before culmination. This pattern of expression mimics that observed for EMLC, suggesting that synthesis of the light chains (and presumably the heavy chain) is coordinated and possibly regulated by similar mechanisms. Furthermore, the pattern of actin transcription is similar to that of the light chain. Therefore, mechanisms that regulate myosin expression may also coordinate the expression of a variety of contractile proteins within the cell. It will be of interest to determine how the structural elements controlling the regulation of the contractile components compare.

Since RMLC synthesis appears to occur during aggregation and culmination, it will be interesting to determine the phenotypes of cells lacking the RMLC. MHC expression mutants are capable of cAMP-directed movement and can aggregate; however, cell movement is much slower than in wild-type cells (66). It is possible that RMLC mutants will have similar motility defects. Alternatively, since light chains are not required for functional ATPase in several systems (39, 57, 63), it is quite possible that a different phenotype will arise. Now that the *Dictyostelium* RMLC has been cloned and sequenced, it can be used in transformation studies to determine the function of the RMLC in vivo and to define the domains within the RMLC that are important for RMLC and myosin function.

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