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ABSTRACT: Myosins and myosin light chain kinases have been isolated from a cloned line of myoblasts (L5/A10) as this cell line undergoes differentiation toward adult muscle. At least three myosin isozymes were obtained during this developmental process. Initially a nonmuscle type of myosin was found in the myoblasts. The molecular weights of the myoblast light chains were 20 000 and 15 000. Myosin isolated from early myotubes had light chains with molecular weights of 20 000 and 19 500. Myosin isolated from myotubes which contained sarcomeres had light chains with molecular weights of 23 000, 18 500, and 16 000. This last myosin was similar in light chain complement to adult rat thigh muscle. Two forms of the myosin light chain kinase activity were detected: a calcium-independent kinase in the myoblasts and a calcium-dependent kinase in the myotubes with sarcomeres. No myosin light chain kinase activity was detected in the early myotubes.

Actin and myosin are the proteins which transduce the energy of ATP hydrolysis into force generation in muscle contraction. Differing forms of these proteins have been demonstrated in many eukaryotic cells (Korn, 1978).

Myosin is a hexamer composed of two polypeptide chains of M_r 200 000 (heavy chains) and four polypeptide varying in molecular weight from 15 000 to 27 000 (light chains). The four light chains of skeletal muscle myosin are further grouped into two classes: 2 mol that are dissociated from the native molecule by denaturation, such as treatment with alkaline solutions, the so-called alkali light chains (Kominz et al., 1959), and 2 mol that can be phosphorylated by a specific enzyme, myosin light chain kinase. One mole of phosphate from ATP is incorporated covalently per mole of light chain, hence the name P light chains (Frearson & Perry, 1975). In nonmuscle and smooth muscle cells the light chains analogous to the alkali light chains of skeletal muscle myosin have molecular weights of 15 000 and the P light chains 20 000. In rat skeletal fast (white) muscle, the alkali light chains have molecular weights of 23 000 and 16 000 and the P light chains 18 500.

In nonmuscle cells as well as in smooth muscle, the interaction between actin and myosin is regulated by phosphorylation of the myosin P light chains. The actin-activated ATPase activity of myosin isolated from proliferative rat myoblasts (Scordilis & Adelstein, 1977), human blood platelets (Adelstein & Conti, 1975), rabbit macrophages (Trotter & Adelstein, 1979), and various smooth muscle cells (Gorecka et al., 1976; Sobieszek, 1977; Chacko et al., 1977) is dependent on phosphorylation of myosin. This phosphorylation is catalyzed by myosin light chain kinase which transfers the γ-phosphate of ATP to a specific amino acid residue on the myosin P light chain.

We report here a study of the changes in contractile proteins of L5/A10 myoblasts as they differentiate, starting with proliferating mononuclear cells and ending with large multinucleated myotubes exhibiting a well-defined sarcomeric structure and spontaneous rhythmic contractions. The findings from the cultured cells are compared to that in adult rat skeletal muscle. The L5/A10 line is a subclone of the L5 line, one of several myogenic cell lines established by Yaffe from primary cultures of newborn rat thigh muscle (Richler & Yaffe, 1970). An important characteristic of L5/A10 cells is that in 10% serum-containing medium they can be repeatedly subcultured and grown to high density without undergoing terminal differentiation, yet without losing their ability to do so. Three to four days after subculture in 1% serum-containing medium these cells are committed to differentiate. The myotubes formed under these conditions, however, tend to disintegrate before forming sarcomeres. This disintegration can be prevented by replacing the medium containing 1% serum with that containing 10% serum (B. W. Uhlendorf, S. Scarpa, and G. L. Cantoni, unpublished results).

In this paper, the developmental changes in myosin and myosin light chain kinase are reported. It is shown that at least three myosin isozymes are expressed during L5/A10 myogenesis. Further, two types of myosin light chain kinases were found, one which does and one which does not require calcium ions for activity.

Materials and Methods

Cell Culture. The L5/A10 myogenic cell line was derived from recloning of the L5 line, obtained from Dr. Marshall Nirenberg. The primary culture had been treated with methylcholanthrene and had been cloned several times before we obtained it (D. Yaffe, personal communication). The L5/A10 cells were maintained by cultivation as monolayers in F14 medium (Vogel et al., 1972), supplemented with 10% fetal bovine serum (Reheis) and neomycin (50 μg/mL). These myoblasts were subcultured at intervals of 3 to 7 days. Three developmental stages of these cells were produced.

Proliferative Myoblasts (PMB).[1] These are uncommitted cells grown to high density in 10% serum in roller bottles (Corning No. 25130) having a growth area of 490 cm^2 per bottle. A total of 52 roller bottles were used to prepare five batches of cells. Myoblasts which had been subcultured from

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[1] Abbreviations used: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; PMB, proliferative myoblasts; EMT, early myotubes; MTS, myotubes with sarcomeres; RTM, rat thigh muscle.

9 to 68 times were used to seed the bottles at 7.5×10^6 to 2.4×10^7 cells per bottle. Every second day the medium (F14 with 10% serum, 150 mL per bottle) was renewed and the cells were harvested on the 8th or 9th day by a trypsin method (Uhlendorf et al., 1973) or a method involving the use of glass beads (Nagle, 1960). No fusion was seen under these conditions. Each roller bottle yielded 1–1.25-mL packed cells. The cell pellets were stored at −75 °C until extraction.

Early Fused Myotubes (EMT). Proliferating cells were subcultured into F14 medium containing 1% serum. The medium was renewed daily. On the 6th or 7th day the cell sheets were harvested by the glass bead method. From 43% to 72% of the nuclei were in myotubes, as determined by counting the nuclei in fixed and stained cell sheets. One batch was prepared from the yield of 10 roller bottles and six more batches were prepared cells grown in a total of 85 large stationary flasks (Costar No. 3150). These cultures yielded about 0.17 mL of packed cells per flask having a growing area of 140 cm^2. The cells had been passaged 11 to 24 times prior to seeding the flasks.

Myotubes with Sarcomeres (MTS). Cell populations were treated as described for the preparation of early fused myotubes until the 6th or 7th day when they were refed with F14 medium containing 10% serum. This medium was renewed daily. On the 7th day the medium was supplemented with 2 mM thymidine in order to prevent overgrowth by variable, mononuclear myoblasts. Further, some of the flasks were additionally supplemented with 1 mM creatine on the last day of culturing. By the 8th or 9th day, cross striations were visible and the myotubes were contracting. The cells were harvested on the 10th day by the glass bead method. The yield was about the same as for the early fused myotubes. Five batches of these myotubes were prepared from 44 flasks. These cells had been passaged 11 to 17 times.

Protein Isolation: Actomyosin, Myosin, Myosin Light Chain Kinase, and Myosin Light Chains. All the isolation procedures were carried out at 4 °C. Cells and muscle tissues were thawed, disrupted, and extracted in 3–5 volumes (1 volume being equivalent to the wet weight of the cells) of high ionic strength solution, buffer A: 0.5 M KCl, 15 mM imidazole–HCl or Tris-HCl, 2.5 mM dithiothreitol (DTT), and 1 mM EDTA, pH 7.5. $Na_4P_2O_7$ (10 mM) and additional DTT (7.5 mM) were added to buffer A for the initial extraction only. The supernate from this slurry was dialyzed overnight against a low ionic strength solution (50 mM KCl, 10 mM imidazole–HCl or Tris-HCl, 2.5 mM DTT, and 0.1 mM EDTA, pH 7.5) and precipitated further by lowering the pH to 6.3 for 1 h. The pellet from this precipitation was suspended in buffer A, made 10 mM in MgATP, and fractionated [by $(NH_4)_2SO_4$ with 10 mM EDTA] into two fractions, 0–30% and 30–55% saturation. The precipitate from the 30–55% fraction was suspended in buffer A, made 10 mM with respect to MgATP, and applied to a Sepharose 4B column, equilibrated and eluted with buffer A. From this column actomyosin, myosin, and the myosin light chain kinase were isolated. More detailed descriptions of the purification procedures have been published previously (Scordilis et al., 1977; Scordilis & Adelstein, 1977, 1978). Myosin light chains were isolated from the 30–55% saturation $(NH_4)_2SO_4$ fraction according to the method of Perrie & Perry (1970).

Myosin Detection and ATPase Activities. The elution profile of myosin from the gel filtration columns was determined by measuring the K^+-EDTA-activated ATPase activities (37 °C) of aliquots of the column fractions in the following assay mixture: 0.5 M KCl, 20 mM Tris-HCl (pH 7.4), 2 mM ATP, and 10 mM EDTA. Inorganic phosphate liberation was measured according to the method of Martin & Doty (1949) as modified by Pollard & Korn (1973). The Ca^{2+}-activated ATPase activites of the pooled myosin fractions were measured in the same assay mixture as above with the substitution of 2 mM $CaCl_2$ for the EDTA.

Myosin Light Chain Kinase Detection and Phosphate Incorporation. The elution profile of the myosin light chain kinase was detected by incubating at 25 °C aliquots of the column fractions with 0.005–0.014 mM isolated rabbit skeletal, canine cardiac, human platelet, or turkey gizzard myosin light chains in an assay mixture that contained 125 mM KCl, 25 mM Tris-HCl or imidazole–HCl (pH 7.4), 12.5 mM $MgCl_2$, 0.6 mM DTT, 0.2 mM $CaCl_2$, and 0.05–0.2 mM ATP (premixed 0.4-mL assay mixture). For the determination of phosphorylation in the absence of calcium ions, 2 mM EGTA was added to the above mixture. The amount of ^{32}P that was incorporated into the light chains was determined by filtration through Millipore filters as described previously (Scordilis et al., 1977; Scordilis & Adelstein, 1978). The incorporation of ^{32}P phosphate was more accurately analyzed on NaDodSO$_4$–polyacrylamide gel electrophoresis of the light chains; 1% NaDodSO$_4$–7.5–10% polyacrylamide gel electrophoresis was carried out according to the method of Fairbanks et al. (1971) with the substitution of diallyltartardiamide for the bisacrylamide in the same molar ratio, according to the method of Anker (1970). The gels were then run, stained with Coomassie Brilliant Blue R, destained, scanned at 584 nm, and cut into 2-mm slices, and each slice was dissolved in 1 mL of an aqueous 2% periodic acid solution at room temperature. Protein concentrations were estimated by the method of Lowry et al. (1951) by using crystalline bovine serum albumin as the standard. Porcine brain calmodulin was purified by the method of Klee (1977).

Results

Myosin. Actomyosin was obtained from the L5/A10 cells at three different stages of differentiation: proliferative myoblasts (PMB), early fused myotubes in which sarcomeres were not evident (EMT), and myotubes in which sarcomeres were evident (MTS). These three actomyosins were compared to each other and to adult rat thigh muscle actomyosin by gel filtration chromatography on Sepharose 4B (Figure 1) which separates actomyosin, myosin, and the myosin light chain kinase. These elution profiles show the relative partition of the proteins actomyosin, myosin, and the myosin light chain kinase. The highest peak of ATPase activity (A_{720}) is due to myosin. The lower peak preceding this peak is due to the ATPase activity of actomyosin. A similar chromatographic pattern has been demonstrated for the adult rat thigh muscle (Scordilis & Adelstein, 1978). The absence of other peaks of ATPase activity is consistent with the idea that little or no proteolysis of the myosin has occurred during the isolation. Evidence consistent with this idea comes from a study of platelet myosin where proteolysis was found to have occurred. The myosin fragment was eluted as a separate peak nearer to $V_0 + V_i$ (Adelstein et al., 1971). No such peak is seen in the present study. Although the various myosins have similar partition coefficients, they represent different isozymic species, as shown below.

Myosin Light Chains. The myosin light chains from the proliferative myoblasts (PMB), the early myotubes (EMT), and the myotubes with sarcomeres (MTS) show three different electrophoretic patterns on NaDodSO$_4$–polyacrylamide gels (Figure 2 and Table I). The apparent molecular weights of the light chains change from a nonmuscle myosin pattern of

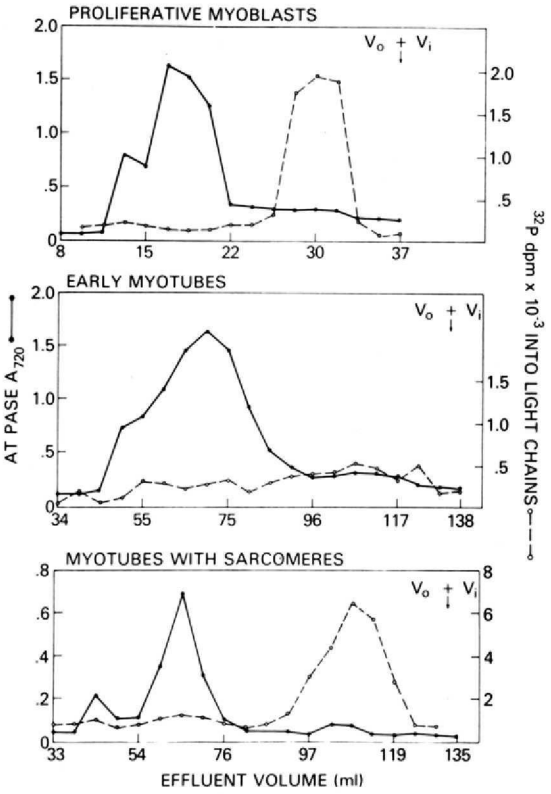

FIGURE 1: Elution profiles from Sepharose 4B chromatography of proliferative myoblast (PMB), early myotubes (EMT), and myotubes with sarcomeres (MTS) actomyosins (30–55% $(NH_4)_2SO_4$ fraction). Samples were applied to the columns (PMB 0.8 mL, 0.9 × 60 cm; EMT 3.2 mL, 1.5 × 100 cm; MTS 2.1 mL, 1.5 × 100 cm), which were equilibrated and eluted with buffer A (see Materials and Methods) at 7.2, 16, and 18 mL/h, respectively. Fractions of 1.2 (PMB), 2.6 (EMT), and 3 mL (MTS) were collected, of which 0.1 mL was used for the K^+-EDTA ATPase assay (A_{720}, ●) and 0.1 mL for the kinase detection assay (○) utilizing canine cardiac myosin light chains. The salt boundaries are indicated as $V_o + V_i$.

Table I: Molecular Weight and Stoichiometry of the Light Chains of Myosin during Development[a]

PMB	EMT	MTS	RTM
20 000 (2.0)*	20 000 (2.1)*	23 000 (1.1)	23 000 (1.5)
15 000 (2.0)	19 500 (1.9)	18 500 (2.2)*	18 500 (1.8)*
		16 000 (0.7)	16 000 (0.7)

[a] All the molecular weights are apparent molecular weights determined from 1% $NaDodSO_4$–7.5% polyacrylamide gels, except for the EMT which is from 1% $NaDodSO_4$–15% polyacrylamide gels. The stoichiometries were determined from the areas under the peaks of scans at 584 nm of the gels. The asterisks indicate the light chains that can be phosphorylated by myosin kinase.

2 mol each of 20 000 and 15 000 daltons for the growing cells (PMB), through an apparent 4 mol of 20 000 daltons for the early myotubes (EMT), to the adult muscle type of roughly 1 mol of 23 000, 2 mol of 18 500, and 1 mol of 16 000 daltons for the myotubes with sarcomeres (MTS). When electrophoresis was carried out on 15% polyacrylamide gels, the light chains of the EMT preparation were resolved into two bands that migrated very closely (Figure 3 and Table I). Only one of these EMT light chain bands was phosphorylatable, that having a molecular weight of 20 000. The second light chain had a molecular weight of 19 500. The light chains of the MTS myosin coelectrophorese with the myosin light chains of the adult rat thigh myosin (data not shown).

Myosin ATPase Activities. The ATPase activities of the column-purified myosins are shown in Table II. In each case, the myosin ATPase activity is activated significantly more in

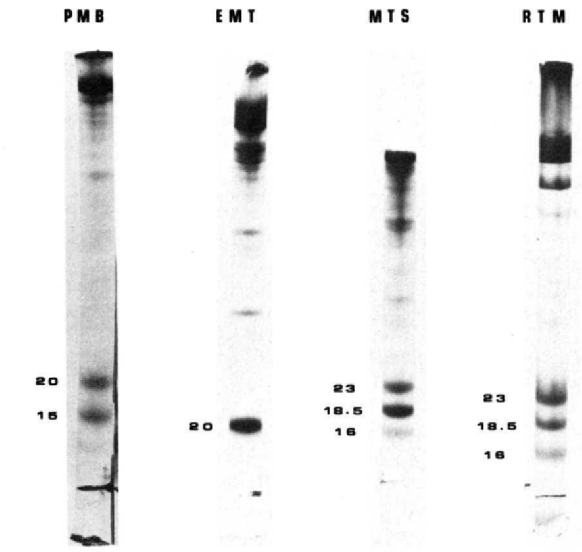

FIGURE 2: 1% $NaDodSO_4$–7.5% polyacrylamide gels of the L5/A10 myosin isozymes from the different developmental stages, as well as rat thigh muscle myosin. PMB, proliferative myoblast with light chains at 20 000 and 15 000 daltons; EMT, early myotube without sarcomeres with light chains of 20 000 daltons on 7.5% gels, but with 20 000 and 19 500 daltons on 15% gels (see Figure 3); MTS, myotubes with sarcomeres with light chains of 23 000, 18 500, and 16 000 daltons; RTM, rat thigh muscle (a 10% gel) with light chains of 23 000, 18 500, and 16 000 daltons. Electrophoresis was from top to bottom.

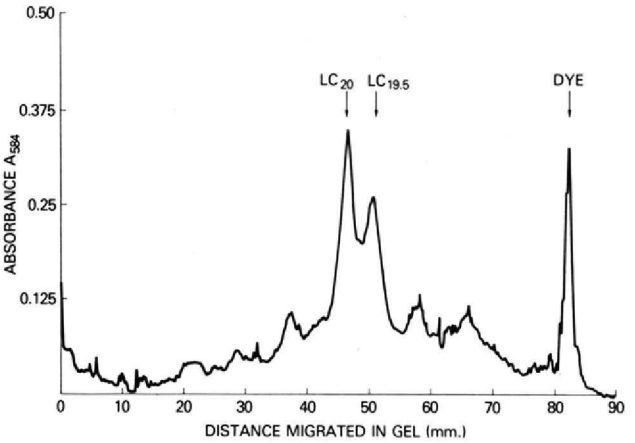

FIGURE 3: A scan of 1% $NaDodSO_4$–15% polyacrylamide gel of the EMT myosin. The two major peaks are the light chains with apparent molecular weights of 20 000 and 19 500. The heavy chain did not enter the gel. Electrophoresis was from left to right.

Table II: Myosin ATPase Specific Activity during Development[a]

	PMB	EMT	MTS	RTM
K^+-EDTA	1.42 ± 0.06	1.29 ± 0.13	1.92 ± 0.31	7.01 ± 0.27
Ca^{2+}	0.22 ± 0.04	0.13 ± 0.02	0.42 ± 0.10	1.39 ± 0.07

[a] The high ionic strength myosin ATPase activities are expressed as the means ± SD ($n = 4$), in μmol of P_i released per min per mg of myosin at 37 °C.

the presence of K^+-EDTA than in the presence of free calcium ions in 0.5 M KCl. The myosin ATPase specific activities are quite similar, although an increasing trend may be discerned toward the adult rat thigh muscle myosin values. It is evident, however, that at a time when the light chain pattern of the MTS myosin is not distinguishable from that of the adult muscle myosin by $NaDodSO_4$–polyacrylamide gel electrophoresis the specific activity of MTS myosin has not yet reached the specific activity of the adult rat thigh muscle myosin. As discussed below, this is consistent with a change

Table III: Kinase Calcium Dependency during Development[a]

kinase	$+Ca^{2+}$	$-Ca^{2+}$
PMB	0.83	0.77
EMT	0	0
MTS	0.67	0.15
RTM	0.87	0.02

[a] The extent of phosphorylation of isolated myosin light chains by the column-purified myosin light chain kinases from the proliferative myoblasts (PMB), the early myotubes (EMT), the myotubes with sarcomeres (MTS), and rat thigh muscle (RTM) in the presence of calcium ions (0.2 mM) and in the presence of EGTA (2.0 mM). All the kinases were purified as described under Materials and Methods and added back to the light chains under the phosphorylation conditions.

in the myosin heavy chain complement.

Myosin Light Chain Kinase. In the isolation procedure used in this study the myosin light chain kinase copurifies with the myosin until they are separated by gel filtration (Figure 1). The kinase peaks separate sharply from the myosin peaks, and no kinase activity is associated with the myosin (Scordilis & Adelstein 1978; Sherry et al., 1978). Both the PMB and MTS elution profiles show peaks of kinase activity (Figure 1). Although these two kinases partition similarly on the Sepharose 4B columns, they have different properties. The PMB myosin light chain kinase does not require free calcium ions (10^{-8} M free Ca^{2+} or greater) for activity, whereas the MTS kinase is largely, but not completely, dependent on calcium ions for activity (see Table III and Figure 4). The degree of stimulation by calcium ions indicates that about 75% of the total kinase activity of the MTS cells is calcium dependent. The rat thigh skeletal muscle is completely dependent on Ca^{2+} for activity (Table III). This is similar to the findings for skeletal muscle myosin kinases isolated from other tissues (Pires & Perry 1977; Yagi et al., 1978). The incorporation of ^{32}P into the cardiac light chain in each of these experiments was verified by $NaDodSO_4$–polyacrylamide gel electrophoresis as shown in Figure 4 for the PMB. There was no significant incorporation of ^{32}P into any other protein.

Gel filtration of the 35–55% ammonium sulfate fraction isolated from early myotubes (EMT) differed from the PMB and MTS in that no peak of myosin kinase activity could be demonstrated. Assays for myosin light chain kinase activity of the eluted Sepharose 4B fractions, using canine cardiac, turkey gizzard, and human platelet myosin light chains as substrates were all negative. No incorporation of ^{32}P phosphate into the myosin in the 30–55% $(NH_4)_2SO_4$ fractions prior to gel filtration was observed. Even when exogenous myosin light chains were added to this fraction, no incorporation could be detected. Moreover, the addition of 10^{-6} M bovine brain calmodulin in the presence of 0.1 mM $CaCl_2$ did not result in myosin kinase activity.

The EMT myosin itself was a suitable phosphate acceptor, as exogenously added kinase could phosphorylate the 20 000-dalton light chain of this myosin. These results tend to rule out the presence of any inhibitor in the EMT myosin fractions. Furthermore, no myosin light chain phosphatase activity could be detected by using ^{32}P-labeled P light chains.

Because proteolysis could play a major role in altering myosin light chain kinase activity, i.e., making it Ca^{2+}-calmodulin independent [see Discussion and Hathaway & Adelstein (1979)] as well as in destroying it, both the PMB and EMT cells were extracted and subjected to gel filtration following ammonium sulfate fractionation in the presence of the following protease inhibitors: 1 mM phenylmethanesulfonyl fluoride, 0.1 mg/mL soybean trypsin inhibitor, 1000 KIU/mL aprotinin, 5 mM EGTA, and 5 mM EDTA. The presence of these inhibitors had no effect on the results shown in Table III. They also had no effect on the molecular weight of the light chains of these two myosins.

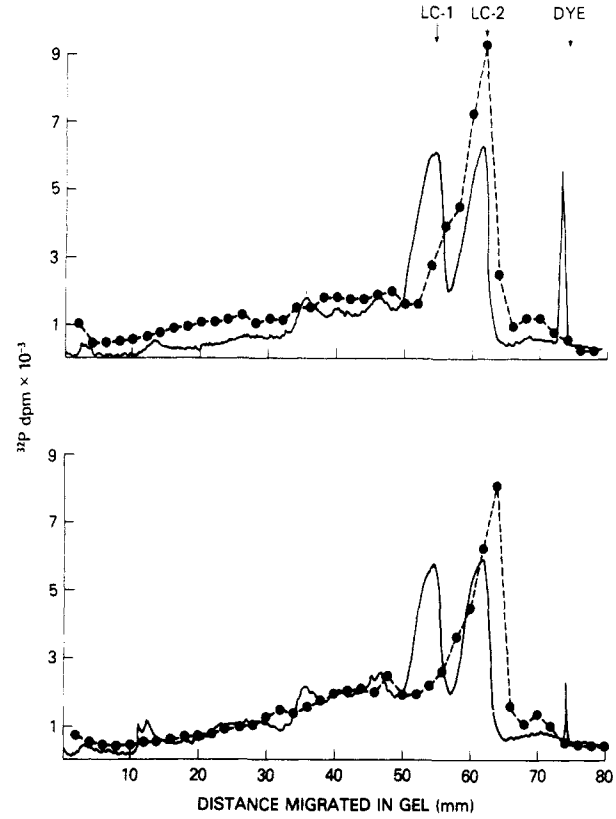

FIGURE 4: Incorporation of ^{32}P into isolated canine cardiac light chains by the column-purified proliferative myoblast myosin light chain kinase in the presence of calcium ions (0.2 mM) (top) and in the presence of EGTA (2.0 mM) (bottom). Identical amounts of the kinase and light chains were incubated under the phosphorylation conditions (see Materials and Methods) and run on 1% $NaDodSO_4$–7.5% polyacrylamide gels. The gels were scanned (solid line) and cut, and the ^{32}P was eluted from each slice counted (●). When corrections for protein loading differences were made, the P light chains incorporated equivalent amounts of ^{32}P in the presence of calcium ions and in the presence of EGTA (0.87 and 0.81 mol of P_i per mol of P light chain, respectively). Electrophoresis was from left to right.

Discussion

The myosin molecule isolated from the L5/A10 clone of myoblasts appears to undergo a differentiation toward the adult form during the myogenic development of these cells in culture. The isozymic forms of myosin (Figure 2) change from a nonmuscle type of myosin (PMB) with light chains of M_r 20 000 and 15 000 to the muscle type of myosin (MTS) with light chains of M_r 23 000, 18 500, and 16 000. In between these two developmental stages, there is a myosin isozyme (EMT) which on 15% polyacrylamide gels appears to have two roughly equal bands of light chains of very close molecular weight (Table I).

These results differ from those previously reported by Chi et al. (1975a,b) and by Yaffe et al. (1977). Chi et al. (1975a,b), using primary cultures of chick breast muscle, describe two types of myosin, one from presumptive myoblasts (comparable to PMB) with light chains of 20 000 and 16 000 daltons. No myosin comparable to EMT myosin was found. Further, their myotube myosin did not contain a third light chain as the MTS myosin does. Yaffe et al. (1977) found two light chains of M_r 23 000 and 17 000 in extracts of differentiated L8 cells, a clonal line of rat origin. The myotubes from

the primary culture, but not those from the L8 culture, also produced a third light chain of M_r 15 000. The presence of this light chain was unexpected since previous studies have not detected an analogous light chain in embryonic chicken pectoralis myotubes in tissue culture (Chi et al., 1975b), embryonic chicken pectoralis muscle (Rubinstein et al., 1977), primary cultures of rat muscle cells, or the myogenic cell line L6 (Whalen et al., 1978). Further, the presence of this light chain in the L5/A10 cloned line suggests that in these cells no neural innervation is necessary for its expression. In neither type of culture was there a transitory light chain produced in early myotubes which was different from the light chain from proliferating and myotube cultures. These differences may be due to the cell types used or perhaps to the conditions used for culture.

Whalen et al. (1978, 1979) have shown that L6 myotubes contain a unique embryonic light chain of M_r 26 000. The results reported here for myosin isolated from cultures of L5/A10 cells are in agreement in that the early myotube stage (EMT) appears to contain a unique light chain (M_r 19 500) not present in the previous PMB or subsequent MTS stage. Similar to the PMB myosin, it also contains a 20 000-dalton phosphorylatable light chain.

The light chains seen in the MTS stage are most likely those of fast myosin. The possibility that slow myosin light chains might also be present, but in much lower quantities, at this stage of development cannot be ruled out. Both Keller & Emerson (1980) and Benfield et al. (1980), using avian species, have provided evidence that both fast and slow myosin light chains are expressed in embryonic muscle.

All of these L5/A10 myosin isozymes apparently maintained the ratio of 4 mol of light chains to 2 mol of heavy chains. Of the 4 mol of light chains, 2 mol from each myosin are homologous to the P light chains of skeletal muscle myosin (Frearson & Perry, 1975); that is, they contain a site which is phosphorylatable by the myosin light chain kinase.

The ATPase specific activities of the myosin isozymes at high ionic strength in the presence of K^+-EDTA and Ca^{2+} serve to indicate the overall similarity of the L5/A10 myosins. However, even though the MTS light chain complement appears to have similar molecular weights and stoichiometries (Table I) to that of the rat thigh muscle myosin, the ATPase activities are not as high. Assuming that the MTS and RTM light chains are indeed identical, then the differences in the ATPase activities of the MTS and RTM may reflect some differences in the heavy chains of these two myosins. It should be recalled that Huszar et al. (1972) found differences in the methylation patterns of fetal and adult myosin heavy chains which indicated that different heavy chains are synthesized during differentiation. Moreover, L6 myosin has been shown to contain a unique, embryonic, heavy chain (Whalen et al., 1979). These results indicate that during myogenic development as many as four different myosin isozymes are being expressed in the L5/A10 line of myogenic cells.

LePeuch et al. (1979) studied the synthesis of myosin light chain kinase in developing chick embryo skeletal muscle. They found that myosin kinase activity was initially low and that it increased rapidly from 2 days before hatching to reach a maximum 3 days after hatching. In contrast, the Ca^{2+}-binding protein calmodulin was found to be synthesized early in embryonic development.

The myosin light chain kinase is expressed in two different forms in the L5/A10 line of myoblasts. The kinase from the PMB cells is Ca^{2+} independent, whereas the kinase from the MTS cultures is mostly Ca^{2+} dependent (Table III). It is probable that the small fraction of mononucleated cells in the MTS cultures contributes the Ca^{2+}-independent myosin light chain kinase. Therefore, it is likely that the muscle-specific (Ca^{2+} dependent) form of myosin kinase is synthesized at the same time as that adult complement of light chains.

The question of the calcium dependency of the myosin kinases from PMB and MTS cells as well as the absence of myosin kinase activity in the EMT cells is complex. Myosin light chain kinase isolated from skeletal (Yazawa & Yagi, 1977; Yagi et al., 1978), smooth (Dabrowska et al., 1978), and cardiac muscle (Walsh et al., 1979) as well as nonmuscle cells such as platelets (Dabrowska & Hartshorne, 1978; Hathaway & Adelstein, 1979) are all dependent on Ca^{2+} and the ubiquitous calcium-binding protein, calmodulin, for activity. In the absence of Ca^{2+} or calmodulin, myosin kinase is inactive. Moreover, the calmodulin binding site can be removed by brief digestion with trypsin (Adelstein et al., 1981). This results in an enzyme that is active in the absence of both calcium and calmodulin.

The presence of a Ca^{2+}-independent kinase in the PMB cells could be due to proteolysis of a Ca^{2+}-calmodulin-dependent myosin kinase, resulting in loss of the calmodulin binding site. There is indirect evidence from work with platelet myosin kinase that this can occur during isolation of myosin kinase. Thus a Ca^{2+}-calmodulin-dependent kinase was isolated from human platelets following addition of proteolytic inhibitors (Dabrowska & Hartshorne, 1970; Hathaway & Adelstein, 1979) in place of a Ca^{2+}-calmodulin-independent kinase (Daniel & Adelstein, 1976).

Proteolysis of the calmodulin binding site cannot be ruled out in the case of the myosin kinase isolated from the PMB cells. However, as noted under Results, addition of broad-spectrum proteolytic inhibitors during the isolation and partial purification of the kinase (including 5 mM EGTA and 1 mM phenylmethanesulfonyl fluoride) had no effect on the Ca^{2+} dependence of the enzyme.

The stage of myogenic development exhibited by the EMT cultures can be considered a pivotal one. Not only is the myosin light chain complement different from both the PMB and MTS myosins but also there is no detectable myosin light chain kinase activity in the preparations. The inability to isolate myosin kinase from these cells might also be ascribed to proteolysis, absence of calmodulin (which would result in inhibition of a calmodulin-dependent kinase), or lack of expression of the kinase gene in these cells. The first possibility seems unlikely since when extracts of these cells were incubated with PMB myosin kinase and MTS myosin kinase, there was no loss of activity. Moreover the addition of proteolytic inhibitors (see above) had no effect. Addition of calmodulin to various fractions of the EMT cells did not result in myosin kinase activity. We therefore tentatively conclude that myosin kinase activity may be lacking during the EMT stage.

Finally, it should be pointed out that there is an essential difference in the culture media between PMB and MTS on the one hand and EMT on the other. The first two preparations were grown in a medium supplemented with 10% fetal calf serum, the latter in a medium containing only 1% fetal calf serum. This change of medium is necessary to trigger differentiation of the myoblast line in vitro. Although it is unlikely that the difference in culture conditions affects the presence or absence of myosin light chain kinase, further studies will be necessary to rule out this possibility.

In summary, it has been demonstrated in this report that L5/A10 myoblasts differentiate toward adult muscle in culture. During this development four myosin isozymes are ex-

pressed if the three different light chain patterns as well as the two different myosin ATPase activities for MTS and RTM are considered. Furthermore two myosin light chain kinase activities are expressed, separated by a stage where no kinase activity can be detected. The changes in these biochemical markers will serve to help us better understand the functional changes implicit in the four myosins and two kinases.

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