Myomesin and M-Protein: Expression of Two M-Band Proteins in Pectoral Muscle and Heart during Development

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ABSTRACT The expression of the myofibrillar M-band proteins myomesin and M-protein was studied in chicken pectoral muscle and heart during differentiation using monoclonal antibodies in a double-antibody sandwich enzyme-linked immunosorbent assay, immunoblotting, and immunocytochemistry. In presumptive pectoral muscle, myomesin accumulated first, increasing from 2% of the adult concentration at day 7 to 70% by day 16 in ovo. M-protein accumulation lagged 6-7 d behind that of myomesin attaining only 40% of the adult concentration in ovo. The molecular masses of myomesin (185 kD) and M-protein (165 kD) remained constant during embryogenesis. In cultured myogenic cells the accumulation and M-band localization of myomesin preceded that of M-protein by 1.5 d.

Chicken heart was shown, in addition to M-protein, to contain unique isoforms of myomesin. In hearts of 6 d embryos, a 195-kD myomesin isoform was the major species; throughout development, however, a transition to a mixture of 195 and 190 kD was observed, the latter being the major species in the adult tissue. During heart differentiation the initial accumulation of myomesin again preceded that of M-protein, albeit on an earlier time scale than in pectoral muscle with M-protein reaching adult proportions first.

Recently we have reported on a new 185-kD protein in the M-band of skeletal muscle myofibrils (1). This brings the number of positively identified M-band proteins to three, the other two being MM-CK (2, 3) and M-protein (4). To avoid multiple nomenclature, it has been agreed to call the 165-kD M-band protein "M-protein" and the recently identified 185-kD M-band protein "myomesin" (1, 5).

Several lines of evidence indicate the functional significance of the M-band which transverses the center of the sarcomere. M-band structural components appear to provide a scaffolding for the alignment and orientation of myosin thick filaments within myofibrils (6-10). Furthermore, there is evidence that the M-band, as well as the Z-disc, are cytoskeletal attachment points and that they assist in maintaining intermyofibril register under the stress of lateral tension imposed on muscle fibers during contraction (11-13).

Of the known M-band proteins, the substructural localization and function of only one has been elucidated. MM-CK, associated with the M4, 4' substriations (14, 15), is present in sufficient quantity to regenerate in isolated chicken myofibrils the ATP required for contraction (16). Attempts have been made to ascertain the roles of other M-band components in myofibrillogenesis (5) and to determine their localization within the substructure of the M-band (14). Now, with monoclonal antibodies (mAb's)[1] available against myomesin and against M-protein (1), we can probe for the structural localization, molecular entities, and functions of these high molecular mass M-band proteins.

In this paper, we examine the presence and appearance during development of myomesin and M-protein in two striated muscles, pectoralis major and heart. Chick heart is particularly interesting because it is known to differ in M-band composition from skeletal muscle to the extent that it contains very little MM-CK, as reflected by the absence of electron-dense material in the M-band (17). Using specific mAb's, it has been possible to verify earlier reports (18, 19) that M-protein is present in heart, although at a lower concentration than in pectoral muscle, and to establish the presence of myomesin as higher molecular mass isoforms. During the differentiation of both pectoral muscle and heart, my-

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[1] Abbreviations used in this paper: ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody; β-ME, β-mercaptoethanol.

omesin is detectable first, suggesting temporally non-coordinate integration into myofibrils.

MATERIALS AND METHODS

Cell Culture: Cell cultures were derived from 11–12-d embryonic chicken pectoral muscle according to established procedures (20) modified by the omission of "pre-plating." Cells were plated on gelatin-coated dishes at a density of 4×10^4 cells/cm² in culture medium containing 10% horse serum and 3.5% embryo extract in Eagle's minimum essential medium with glutamine and antibiotics.

Extracts of Tissue and Cell Cultures: Pectoral muscle from White Leghorn embryos or adult chickens was excised and immediately frozen in liquid nitrogen to reduce proteolytic degradation. Phosphate-buffered saline (PBS) was added at an appropriate weight per volume ratio to keep the final protein concentration as concentrated as possible (~10 mg/ml) and the tissue was homogenized with a Polytron homogenizer (Kinematica, Lucern, Switzerland). Cell cultures at various stages of differentiation, which had been washed with PBS and stored at −20°C, were scraped from the dishes in the smallest possible volume of PBS. From this point on tissues and cells were treated identically. SDS and β-mercaptoethanol (β-ME) were added to concentrations of 0.3% and 1%, respectively. After mixing with a Pasteur pipette, 0.12 ml of a DNase-RNase solution (1 mg/ml pancreatic DNase I, 0.5 mg/ml pancreatic RNase [both from Sigma Chemical Co, St. Louis, MO], 50 mM $MgCl_2$, 0.5 M Tris-HCl, pH 7) was added per milliliter of homogenate. Subsequent procedures were performed at room temperature. The homogenates were mixed, sonicated for 15 s, and solubilized by bringing the SDS concentration to 2%. After centrifugation in a microcentrifuge for 5 min, only a minute insoluble pellet was observed. The protein concentration of the extracts based on the absorption of tryptophan and tyrosine residues was determined with a fluorescence spectrophotometer (Model MPF-44A, Perkin-Elmer Corp., Küsnacht ZH, Switzerland) at an excitation wavelength of 280 nm and an emission wavelength of 330 nm using bovine serum albumin (BSA) as a standard. Aliquots of the extracts were immediately diluted 1:2 in sample buffer for PAGE and boiled for 10 min. All extracts were stored at −20°C.

Antibodies: The mAb's against the 185-kD myomesin (B4 and B5) and the 165 kD M-protein (A5 and A6) have been previously characterized (1). Affinity-purified rabbit antibody against M-protein, later found to react with both myomesin and M-protein, was prepared in this laboratory by Dr. T. Doetschman according to Strehler et al. (18, 19). Monospecific polyclonal antibodies were obtained by adsorption of antisera to antigen containing nitrocellulose strips and elution according to the procedure of Olmsted (21), which was adapted for the purification of specific antibodies against myomesin and M-protein in our laboratory (32). Since the nitrocellulose strips with M-protein used to purify the antibodies contained degradation products of myomesin, the myomesin-reacting antibodies in the M-protein eluate were adsorbed by incubating the eluate with the myomesin strips.

Rabbit anti-mouse IgG was peroxidase conjugated as described (22). Fluorescein isothiocyanate–labeled sheep anti-mouse IgG, rhodamine-labeled sheep anti-mouse IgG, and rhodamine-labeled goat anti-rabbit IgG were purchased from Cappel Laboratories, Cochranville, PA.

Electrophoresis and Immunoblotting: Extracts were electrophoresed on 5% SDS polyacrylamide gels, electrophoretically transferred to nitrocellulose membranes (23), and reacted with mAb's as previously described (1).

Double-Antibody Sandwich Enzyme-linked Immunosorbent Assay: The double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) (24) was adapted for the detection of myomesin and M-protein as follows. PVC microtiter wells (Costar, Cambridge, MA) were coated with a saturating amount (1.4 μg/ml) of polyclonal affinity-purified antibody against both myomesin and M-protein for 2 h and blocked with 1% BSA, 0.2% NaN₃ in PBS for 30 min. Since the extracts contained 2% SDS, preliminary experiments were necessary to establish that 0.2% SDS in the presence of 2% Triton X-100 did not interfere with the assay. Thus, the extracts were initially diluted 1:10 in 2.2% Triton X-100, 1% β-ME, 1% BSA, 0.02% NaN₃ in PBS to a final concentration of 0.2% SDS, 2% Triton X-100, and 1% β-ME. Subsequent dilutions were made in 0.2% SDS, 2% Triton X-100, 1% β-ME, 1% BSA, 0.02% NaN₃ in PBS. Extracts at the appropriate dilutions were incubated in the wells overnight at 4°C. Saturating amounts of mAb culture supernatants were added for 2 h followed by peroxidase-conjugated rabbit anti-mouse IgG diluted 1:1,000 for 1 h. Alternatively, a 10-fold more sensitive assay was obtained by replacing the peroxidase-conjugated rabbit anti-mouse IgG with biotinylated anti-mouse IgG followed by avidin:biotinylated peroxidase complex according to the manufacturer's instructions (Vector Laboratories, Inc., Burlingame, CA). The assays were developed and terminated as described

(1). Myomesin and M-protein content were determined relative to that in adult pectoral muscle per milligram protein by calculating the displacement of curves obtained from serial dilutions of extracts compared to the dilution curve obtained for adult pectoral muscle.

Immunocytochemistry: Cell cultures were fixed in 3% paraformaldehyde in PBS for 30 min, permeabilized with 0.2% Triton X-100 for 5 min, and reacted overnight with a mixture of anti-myomesin B4 hybridoma culture supernatant and monospecific polyclonal anti-M-protein antibodies (~2 μg/ml) at 4°C. Fluorescein isothiocyanate-labeled sheep anti-mouse IgG (1:200) and rhodamine-labeled goat anti-rabbit IgG (1:200) were used as secondary antibodies to stain the bound mAb's and polyclonal antibodies, respectively.

mAb's against M-protein did not readily react with cells fixed with paraformaldehyde even after SDS treatment (1). Thus an alternative fixation procedure had to be used. Specific M-band binding could be detected after the cells were fixed either with 20% ethanol, 1% acetic acid, and 2% formalin or with 100% ethanol.

RESULTS

Analysis of Myomesin and M-Protein Isoforms in Pectoral Muscle and Heart of Adult Chickens

The specificities of the mAb's against the M-band proteins myomesin and M-protein in adult chicken pectoralis major have been documented (1). To determine the presence of myomesin and/or M-protein in heart, and also to compare and characterize these proteins, extracts of pectoral muscle and heart were electrophoresed on SDS polyacrylamide gels and immunoblotted with mAb's against myomesin or against M-protein (Fig. 1). In heart (Fig. 1 a), the mAb against myomesin stained a band of 190 kD and a minor band of 195 kD, both higher than the 185-kD band found in pectoral muscle (Fig. 1 b), indicating the existence of myomesin as isoproteins. A band of 185 kD was never detected in heart extracts. M-protein was detected as a band of 165-kD in both heart and pectoralis (Fig. 1 a', 1 b'), Occasionally lower molecular mass bands occurred (Figs. 2 and 3) representing degradation products which are difficult to control even with protease inhibitors. It is highly unlikely that these bands represent other proteins which cross-react with the mAb's, since the same blotting patterns were seen with the purified fractions of myomesin and M-protein (peaks I and II, respectively, in Fig. 4 of reference 1) and with low ionic strength extracts of pectoral myofibrils (Fig. 5 of reference 1). Among all the striated muscles examined, including anterior latissimus dorsi, posterior latissimus dorsi, and medial adductor (data not shown), only heart exhibited myomesin bands larger than 185 kD, whereas M-protein when present consistently appeared as a band of 165 kD.

Myomesin and M-Protein Isoforms in Pectoral Muscle and Heart during Development in Ovo

Since the earliest myosin detectable in the somitic myotome, the precursor to skeletal muscle, specifically reacts with anti-cardiac myosin heavy chain (25), it was of interest to know if isoforms of the myosin-associated protein myomesin also appear during development. To identify the isoforms of myomesin and/or M-protein present in chicken pectoral muscle and heart during development, embryonic tissue extracts were resolved by SDS PAGE and immunoblotted. As shown in Fig. 2(7–Ad), the patterns of the proteins extracted from presumptive pectoral muscle changed during development and, most notably, myosin heavy chain increased dramatically. The quantity of myosin heavy chain in extracts of day 16 and older pectoral muscle limited the amount of
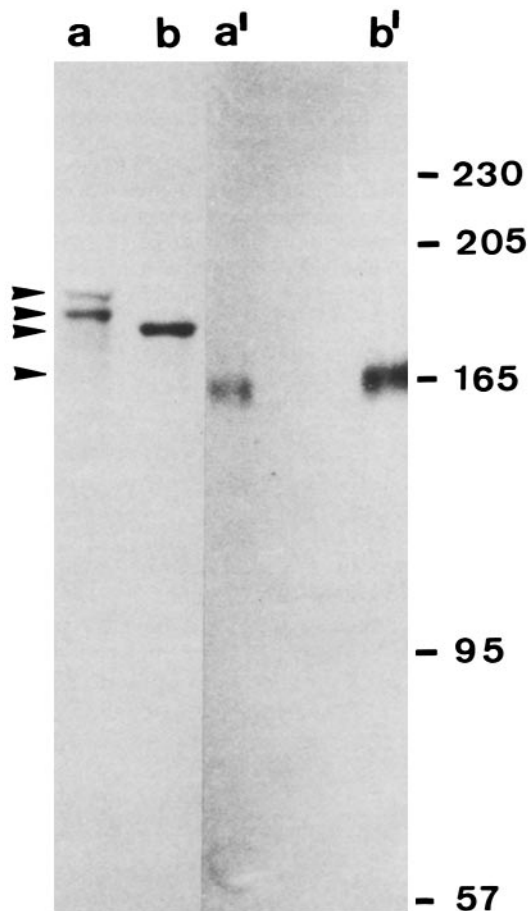
FIGURE 1 Immunoblots of adult pectoral muscle and heart with mAb's against myomesin and M-protein. Extracts, 80 µg total protein per lane, of pectoral muscle (b and b') and heart (a and a') were resolved by 5% SDS PAGE, electrophoretically transferred to nitrocellulose paper, and reacted with mAb's as described in Materials and Methods. (a and b) Reaction with mAb B5 against myomesin. (a' and b') Reaction with mAb A6 against M-protein. Protein standards co-electrophoresed for molecular mass estimations were fibronectin (230 kD), myosin (205 kD), glycogen debranching enzyme (165 kD), phosphorylase b (94 kD), and pyruvate kinase (57 kD). Arrowheads mark the positions of immunoblotted bands with calculated molecular masses of 195, 190, 185, and 165 kD.

extract that could be loaded on gels to ~60 µg protein per lane. Fig. 2(7'-Ad') shows the reaction of anti-myomesin mAb B5 with a nitrocellulose replicate of extracts from day 7 to adult tissue. In extracts derived from day 12 on, mAb B5 reacted with a band of 185 kD. Lower bands represent degradation products (1). In Fig. 2(7"-A") anti-M-protein mAb A6 was reacted with a second nitrocellulose replicate. As with the mAb against myomesin, from day 12 on mAb A6 reacted solely with a band of 165 kD. No bands were detectable at day 7 with mAb's against either protein. Due to the lower concentration of myosin in the extracts from early embryos (days 7–10), up to 200 µg of protein could be loaded without distortion of the 185 kD band. In immunoblots of such gels, myomesin and M-protein could be detected as faint bands of 185 kD and 165 kD, respectively (data not shown).

Embryonic heart tissue was similarly examined for isoproteins of myomesin and M-protein (Fig. 3). Fig. 3(7–Ad) shows the protein staining pattern of the heart extracts. In immunoblots of embryonic heart extracts, mAb B5 against myome-

sin strongly reacted with a band of 195 kD in extracts of 7 and 11 d hearts (Fig. 3[7'-Ad']). By day 18, the 195-kD band had decreased in intensity and the 190-kD band predominated. As was found with presumptive pectoral muscle, mAb A6 against M-protein stained identical bands of 165 kD in extracts of hearts at all developmental ages examined (Fig. 3[7"-Ad"]).

Accumulation of Myomesin and M-Protein during Embryonic Development

One approach to determining the roles of myomesin and M-protein in myofibrillogenesis is to determine when they appear during development. While immunoblots give an indication of the presence of myomesin and M-protein at different developmental stages, they are not quantitative and give only rough estimates of the amounts of proteins present. A quantitative assay can be carried out with the ELISA. Myomesin and M-protein, however, are present in tissue extracts in such small concentrations that direct application of extracts to microtiter wells as in a standard ELISA resulted in a high background reaction, effectively lowering the signal to noise ratio and reducing the sensitivity of the assay. Therefore a double-antibody sandwich ELISA was developed to quantitate the amounts of myomesin and M-protein present in developing pectoral muscle. Tissue extracts were added to microtiter wells coated with polyclonal antibody which reacts with both myomesin and M-protein (1). These immobilized antibodies selectively bound myomesin and M-protein which could then be readily detected with the appropriate mAb followed by a peroxidase-conjugated secondary antibody.

Fig. 4 illustrates the results obtained for several of the extracts tested in a double-antibody sandwich ELISA in which twofold dilutions of extracts of pectoral muscle from embryos from day 7 through hatching and from adult chickens were incubated in wells coated with polyclonal antibody. Bound myomesin (Fig. 4A) was detected with a mixture of anti-myomesin mAb's B4 and B5. Bound M-protein (Fig. 4B) was detected with anti–M-protein mAb's A5 and A6. The use of multiple mAb's which recognize different antigenic determinants on the same protein (1) increased the sensitivity of the assay. Myomesin and M-protein content as a function of developmental age were calculated relative to the concentrations in adult pectoral muscle per milligram of protein (Fig. 5). Significant concentrations of myomesin began to accumulate at day 7, plateaued at 20% of adult values from days 9 through 11, and then rose to 70% by day 16 where it remained through hatching. M-protein, on the other hand, was barely detectable at the early days and did not begin to appear in significant quantities until day 12 when it gradually increased to 40% of adult values on day 20. The expression of myomesin and M-protein during the development of pectoral muscle appears to be differentially regulated in that the accumulation of myomesin preceded that of M-protein by 6–7 d.

The accumulation of myomesin and M-protein in heart during embryogenesis was also quantitated in extracts by means of a double-antibody sandwich ELISA (Fig. 6). Already by day 6 myomesin was present at ~25% of the adult concentration, but showed, in contrast to pectoral muscle, only an increase to ~50% before hatching; a further increase in accumulated antigen occurred after hatching. Also in contrast to pectoral muscle, in heart M-protein was detectable at a
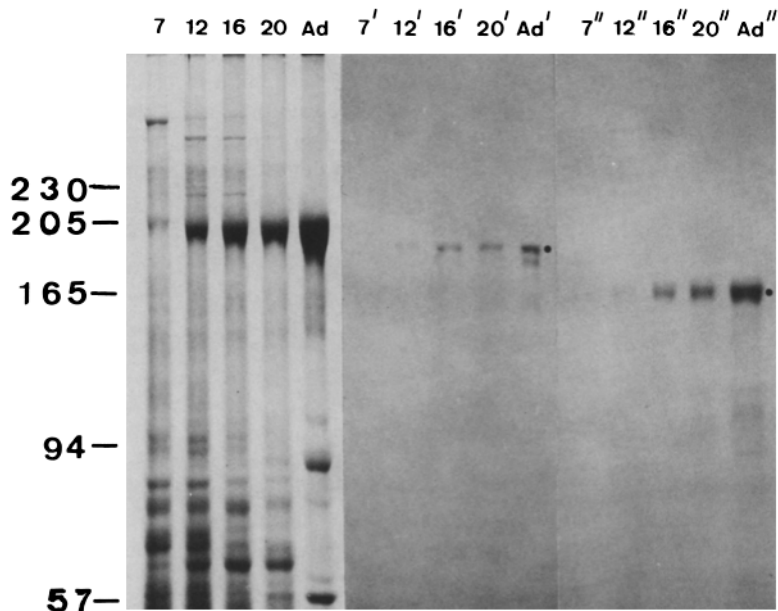
FIGURE 2 Reactions of embryonic pectoral muscle extracts from day 7 in ovo to the adult with mAb's against myomesin and M-protein. (7–Ad) Coomassie Blue–stained 5% polyacrylamide gel. Nitrocellulose replicates were reacted with mAb B5 against myomesin (7'–Ad') or with mAb A6 against M-protein (7"–Ad"). 60 μg total protein was applied to each lane. Numbers indicate the days of incubation in ovo (7–20). Ad, adult pectoral muscle. Molecular mass markers were the same as those in Fig. 1. Myomesin and M-protein were always detected as bands of 185 kD and 165 kD, respectively, and are indicated by dots.
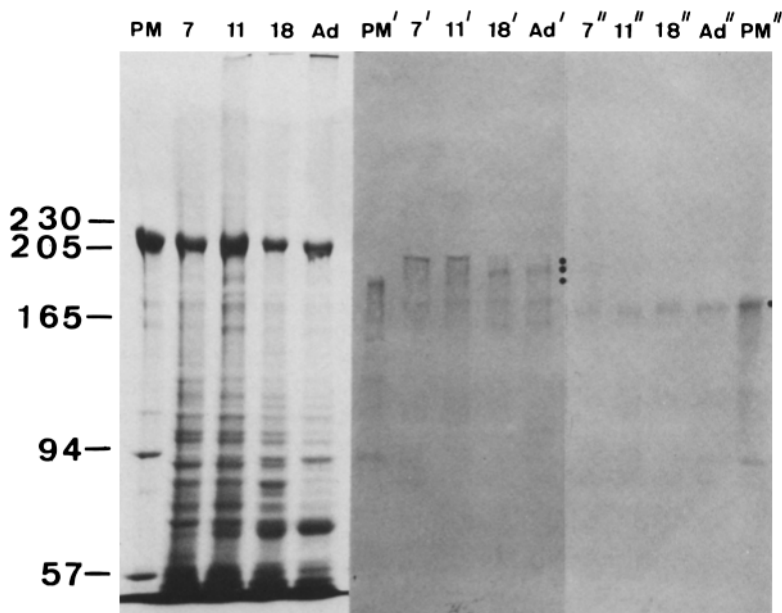

FIGURE 3 Reactions of embryonic heart extracts from day 7 in ovo to adult with mAb's against myomesin and M-protein. (PM—Ad) Coomassie Blue–stained gel. 120 μg total protein was applied to each lane containing heart extracts. 40 μg total protein was applied to lanes containing adult pectoral muscle extract (PM). Otherwise procedure and designations were the same as in Fig. 2. Significant bands are indicated by dots as in Fig. 1.

higher concentration (5%) at day 6 relative to adult heart and showed an almost linear increase to adult levels by 4 d after hatching.

The finding that both M-band proteins accumulated earlier in embryonic heart than in pectoral muscle is most likely a reflection of earlier maturation and functioning of heart tissue. For explaining the faster increase in accumulation of M-protein compared to pectoral muscle, it has to be considered that the adult heart concentration of M-protein was only 13% of that found in adult pectoral muscle (data not shown).

Appearance of Myomesin and M-Protein in Myogenic Cells in Culture

From earlier experiments with the polyclonal antibody made against M-protein (5), now known to react with both myomesin and M-protein, it can be deduced that at least one of these two M-band proteins is being synthesized by embryonic pectoral cells in culture.

Now with mAb's it has become possible to specifically quantitate the accumulation of myomesin and of M-protein in cultures of myogenic cells. Mononucleated myogenic cells were cultured and extracts were made from days 1 to 9. The amounts of myomesin and M-protein in the extracts were determined in a double-antibody sandwich ELISA as described (see legend to Fig. 4). Cell cultures, fixed at the same time points and stained with Giemsa, were used to determine the fusion index. In 24-h cultures, a few multinucleated cells were already present and a low level of myomesin could be measured, while M-protein was barely detectable (Fig. 7).

Although fusion occurred rapidly during the second day of culture due to a relatively high cell density, myomesin concentration rose at a moderate rate and almost no increase in M-protein concentration was observed. In 2 to 4 day cultures, the rise in myomesin became accelerated with myomesin reaching a maximum concentration on day 6. The accumulation of M-protein lagged behind that of myomesin by 1½ d but again the maximum concentration was attained on day
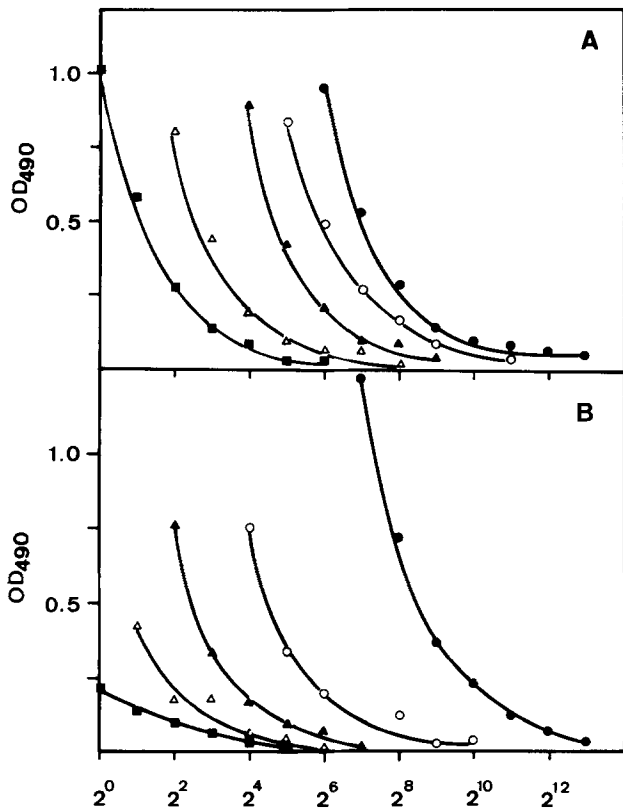
FIGURE 4 Titration of embryonic pectoral muscle extracts in a double-antibody sandwich ELISA for the detection of myomesin (A) and M-protein (B). Muscle tissue was extracted in 2% SDS/1% β-ME and twofold serial dilutions of the extracts were added to microtiter wells coated with polyclonal antibodies recognizing both myomesin and M-protein as described in Materials and Methods. The initial protein concentration of the extract at 2°C was 1 mg/ml. Bound myomesin was detected by a combination of mAb's B4 and B5 (A), whereas bound M-protein was detected by a combination of mAb's A6 and C2 (B). The curves shown are for extracts from 7-d (■), 8-d (△), 10-d (▲), 14-d (○) embryonic muscles and from adult (●) pectoral muscle. Each point represents the average of duplicate values. Values obtained for adult chicken liver extract, known not to contain either protein (data not shown), were subtracted as background.

6. Overall from days 1 to 6 there was an 18-fold increase in the concentration of myomesin and a 78-fold increase in M-protein. The decline in the amounts of myomesin and M-protein after 6 d in culture could be due to proliferation of fibroblasts and/or detachment of differentiated myotubes from culture dish.

When the relative amount of myomesin was compared with that of M-protein, the ratio of myomesin to M-protein rose from 9 in day 1 cultures to 20 in newly formed myotubes on day 2, and then fell to 9 on day 3 as the myotubes matured. In older cultures, the myomesin to M-protein ratio stabilized at day 2 due to an increase in the relative amount of M-protein. This pattern of accumulation of myomesin and M-protein was observed in three independent experiments and is representative of the accumulation of these M-band proteins.

To rule out the possibility that the particular antigenic determinants measured in the foregoing experiments were masked at earlier stages or not present on an isoform of the protein which appeared earlier, each of three mAb's against

myomesin and four mAb's against M-protein was used separately in the double-antibody sandwich ELISA to detect the accumulation of myomesin or M-protein in cell culture extracts. All of the antigenic determinants accumulated in parallel from day 1 through day 4 in culture (data not shown), indicating that each antigenic determinant is representative of the whole molecule.

A relevant question following from the ELISA data on the appearance and accumulation of myomesin and M-protein in myogenic cell cultures concerns the distribution of these two M-band proteins in individual cells. Does myomesin accumulate earlier than M-protein in all cells or are there different populations of cells, one differentiating earlier which accumulates only myomesin and another differentiating later which accumulates both M-band proteins? To answer this question, individual cells at different stages of differentiation were examined in dual immunofluorescence experiments using anti-myomesin mAb B4 and a monospecific polyclonal antibody against M-protein which was purified as described in Materials and Methods. A monospecific anti-M-protein polyclonal antibody was used in these experiments because it
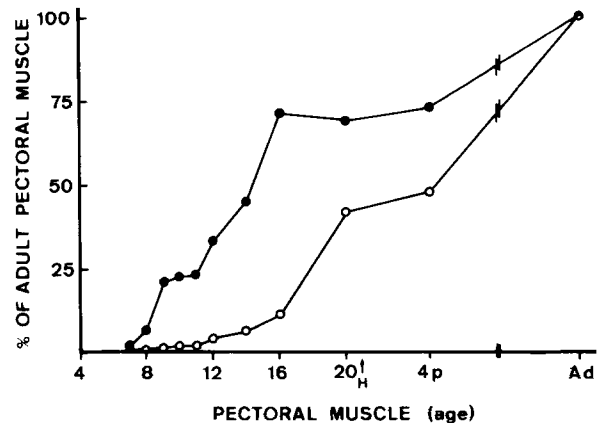

FIGURE 5 Appearance of myomesin (●) and M-protein (○) in embryonic pectoral muscle during development. Extracts of pectoral muscle from 7 to 20 d in ovo, 4 d posthatch (4p), and adult (Ad) were assayed by titration in a double-antibody sandwich ELISA as described in Materials and Methods. Representative curves are shown in Fig. 4. Myomesin and M-protein content were determined relative to that in adult pectoral muscle per milligram of protein and expressed as percent of adult pectoral muscle. H, hatched.
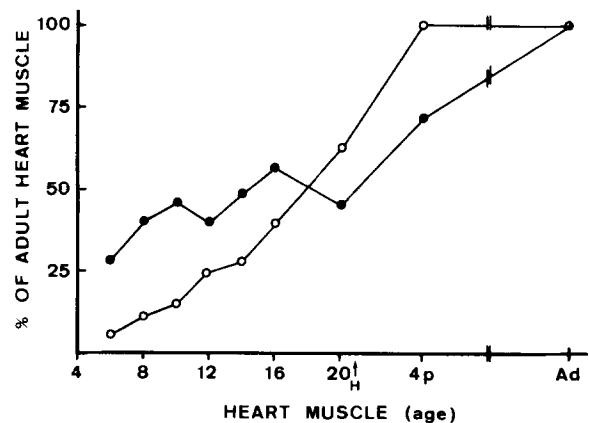

FIGURE 6 Appearance of myomesin (●) and M-protein (○) in embryonic heart during development. The content of myosin and M-protein in embryonic heart relative to that in adult heart was determined for the ages indicated as described in Figs. 4 and 5.
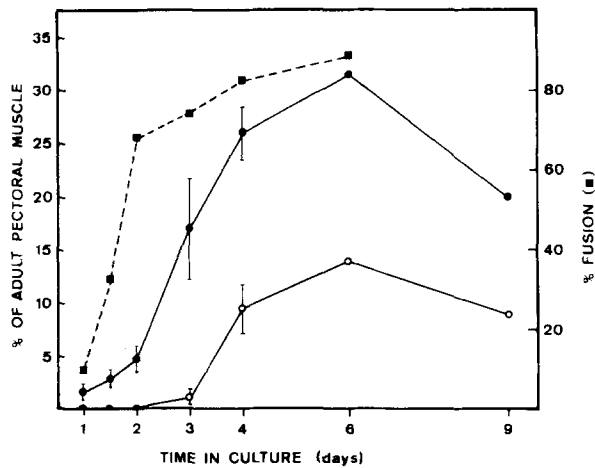
FIGURE 7 Accumulation of myomesin (●) and M-protein (○) in myogenic cell cultures. Myogenic cells from pectoral muscle from 11–12-d chicken embryos were cultured for 1–9 d and then extracted with 2% SDS, 1% β-ME as described in Materials and Methods. The content of myomesin and M-protein relative to that in adult pectoral muscle (100%) was determined by titration of the extracts in a double-antibody sandwich ELISA as described in Materials and Methods. The data is drawn from three separate experiments. The points for 1, 2, and 3 d represent the average of three independent measurements; those for 1.5 and 4 d represent two separate experiments; and the points for 6 and 9 d are from a single experiment. When possible, the standard deviation is indicated by bars. Background values were determined in wells in which extract was omitted. ■, fusion index representing percent of nuclei in myotubes.

stained M-protein more intensely than did the mAb's and facilitated the procedure for dual labeling by indirect immunofluorescence. Its monospecificity was tested in two ways. Immunoblots with chicken pectoral muscle extract showed a strong reaction with the 165-kD band as expected and a very faint band at 185 kD indicating the presence of minor contaminating anti-myomesin antibodies. In indirect immunofluorescence assays, the anti–M-protein antibodies brightly stained the M-band of pectoral myofibrils while negligible M-band staining of anterior latissimus dorsi myofibrils, shown with mAb's not to contain M-protein, could be detected (data not shown). Thus, staining of this intensity was considered to be negative for M-protein and the purified antibody could be used as a monospecific reagent for the detection of M-protein.

The results of immunofluorescence experiments in which myogenic cells in culture were examined for the presence of myomesin and M-protein are shown in Fig. 8. The majority of mononucleated cells were negative for both M-band proteins although occasional myoblasts exhibited cross-striations indicative of the presence of both myomesin and M-protein (Fig. 8a) and a few were positive for myomesin alone. Cells that were negative for myomesin and positive for M-protein were never observed. After 48 h in culture, most of the myogenic cells had fused. In many of these newly formed myotubes, mAb against myomesin gave a pattern of punctate fluorescence (Fig. 8b) suggesting that the myofibrils were not yet in register and the M-bands not sufficiently aligned for a cross-striated pattern of fluorescence. M-protein was never detectable until myomesin appeared in a cross-striated pattern, only then could M-protein also be detected in the striations (Fig. 8b). As myotubes became thicker and myofibrils could be seen by phase-contrast microscopy, the intensity

of M-protein staining also increased until it equaled that obtained for myomesin (Fig. 8, c and d).

Even when the labels on the secondary antibodies were switched, the relative intensities of staining for myomesin and M-protein remained the same at different stages of differentiation. Thus, differences in intensity could not be attributed to the particular secondary antibody used. Identical results were obtained with anti–M-protein mAb's and monospecific anti-myomesin polyclonal antibodies as the dual-labeling reagents, although as mentioned the staining with anti-M-protein mAb's was not as intense as that obtained with the monospecific anti–M-protein polyclonal antibodies.

These results confirm the ELISA data that M-protein is not accumulated as early in myogenesis as myomesin. In addition, it can be concluded that while individual myogenic cells may initially contain only myomesin, all differentiated cells eventually contain both myomesin and M-protein.

DISCUSSION

Using mAb's specific for the M-band proteins myomesin and M-protein in chicken pectoral muscle in conjunction with immunoblot and ELISA assays, we have shown that both proteins are also present in heart, however the protein bands in heart recognized by mAb's against myomesin (195 kD and 190 kD) are of higher molecular masses than in pectoral muscle (185 kD), whereas the protein band in heart recognized by anti–M-protein mAb's, although less intense, has a molecular mass identical to that in pectoral muscle (165 kD).

Only the heart forms of myomesin changed in mobility during embryonic development. The transition from a band of 195 kD to one of 190 kD in heart and the presence of a 185-kD band in pectoral muscle could have several explanations. These bands may represent isoforms of myomesin that are products of different genes which are regulated by different switching mechanisms or they may be products of the same gene which are subsequently differentially processed. An increase in Ca⁺⁺-activated protease activity during development (26) could be responsible for modulation of the heart isoforms. Alternatively, the 190-kD and/or the 185-kD bands may be degradation products of the 195-kD band obtained as a result of the extraction procedure. Support for this hypothesis comes from evidence for endogenous muscle proteases (27, 28) and the high susceptibility of both myomesin and M-protein to degradation (see Fig. 5 of reference 1). However, evidence against this hypothesis is seen in Fig. 1 where heart myomesin and M-protein appear to be less susceptible to endogenous protease activity than the corresponding proteins in pectoral muscle. Even stronger is the evidence that a band of 185 kD is never detected among heart myomesin degradation products. If high levels of proteolytic activity were responsible for the degradation of myomesin in pectoral muscle to a band of 185 kD, then the heart myomesin isoforms extracted in the presence of pectoral muscle should undergo a similar fate. However, immunoblots of adult pectoral muscle and heart extracted together contained all three isoforms and evidence for degradation of the heart 195-kD and 190-kD proteins was not detectable (data not shown). It has been shown that mAb's occasionally react with completely different proteins bearing similar antigenic sequences (29). In this instance several pieces of evidence (data not shown) tend to rule out this possibility: (a) mAb's against myomesin stain the M-band region of heart myofibrils in immunofluorescence; (b) all
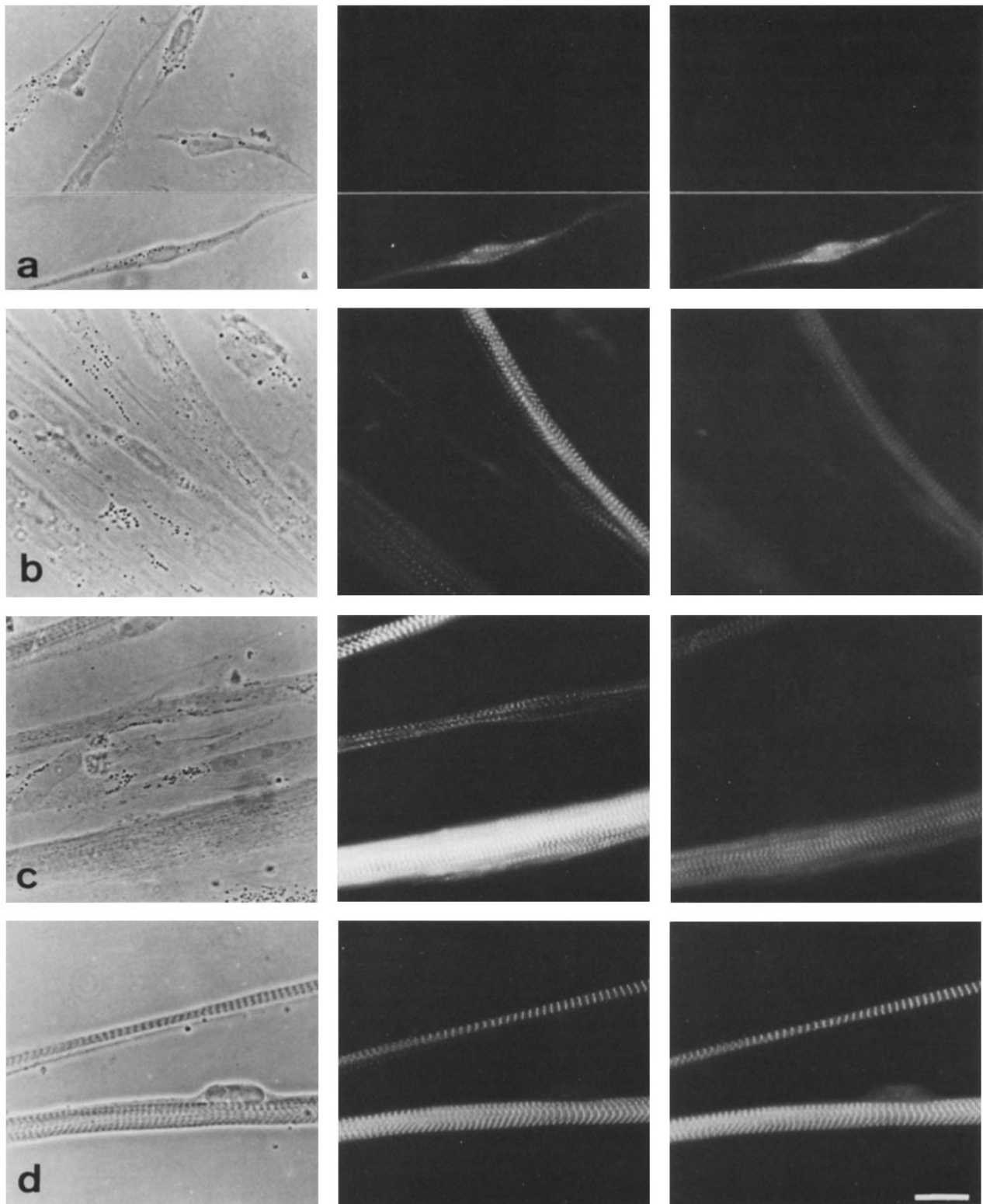
FIGURE 8 Dual immunofluorescent labeling of myomesin (center) and M-protein (right) in myogenic cells after (a) 1, (b) 2, (c) 3, and (d) 10 d in culture. (Left) Phase-contrast pictures. Paraformaldehyde-fixed cultures were incubated overnight with monoclonal anti-myomesin B4 antibodies and polyclonal affinity-purified rabbit anti-chicken M-protein antibodies (4 μg/ml). This was followed by incubation (30 min) with fluorescein isothiocyanate–labeled sheep anti-mouse IgG (for myomesin) and rhodamine-conjugated goat anti-rabbit IgG (for M-protein). Bar, 20 μm.

mAb's demonstrate the same relative binding to both pectoral muscle and heart in ELISA; and (c) the polyclonal antibody against myomesin and M-protein also recognizes both heart 190-kD and 185-kD bands with high intensity. Thus the

evidence suggests that the anti-myomesin mAb is recognizing isoforms of myomesin in heart and pectoral muscle. The origin of these myomesin isoforms remains to be resolved.

A molecular mass of 165 kD for M-protein appears to be

characteristic of both pectoral muscle and heart throughout development. If other molecular mass isoforms exist, they would have to be missing the antigenic determinants recognized by the available mAb's and escape detection. Although all detectable M-protein seems to be of the same apparent molecular mass, it is still possible that the M-protein band could consist of isoforms detectable only at the level of the primary sequence and not be distinguishable in our assays.

Another distinction between the M-band proteins in pectoral muscle and heart is that while both types of muscle contain approximately the same amount of myomesin per milligram of total protein, heart fibers contain only 13% as much M-protein. This observation was indicated by immunoblots and confirmed by relative ELISA quantitation. In immunofluorescence experiments, it has been shown that both myomesin and M-protein are co-localized in all fibers (data not shown) indicating that heart myofibrils function with lower levels of M-protein. Alternatively, if there are fewer myofibrils in heart per milligram of total protein, then the findings could also be interpreted that heart myofibrils contain an excess of myomesin compared to pectoral muscle myofibrils.

The crucial question is, What are the functions of these two M-band proteins? To determine potential roles of myomesin and M-protein in the assembly of myofibrils, knowledge of their appearance during development may provide some clues. During the differentiation of pectoral muscle myomesin begins to accumulate in appreciable quantities several days before M-protein. From Fig. 5 it is not possible to make a direct comparison of absolute quantities of myomesin and M-protein. However, on the basis of rough calculations from data in reference 1, there appears to be approximately 2.5- to 3-fold more M-protein in adult pectoralis major than myomesin. If this holds true, there would still be a lag in the actual amount of M-protein accumulated relative to myomesin. This pattern of non-coordinate accumulation is also distinctive in heart. Just as striking is the earlier and more dramatic accumulation of myomesin in cultured cells on a more compacted time scale which one would expect from cells which display greater synchrony of differentiation. The finding that M-protein is present in 1–2 d cultures in such small quantities (<0.3%) relative to the adult would suggest that its apparent absence from M-bands in myofibrils containing myomesin is due either to its actual absence or to the presence of levels below the detectability of the assay rather than to the accumulation of non-assembled M-protein.

Myomesin appears to accumulate relatively early in myofibrillar assembly preceding the rise in accumulation of myosin light chains in embryonic pectoral muscle (based on extrapolation of data from reference 30), however the accumulation of myomesin correlates well with the transition from both fast and slow light chains to exclusively fast light chains and the assembly of myosin light chains into complete myosin molecules (30, 31). Sequential appearance in sarcomeres of myosin, myomesin, and further delayed M-protein was demonstrated in a recent double-immunofluorescence study of differentiating synchronized myogenic cells (A. Fulton, personal communication). Although there is little data on the accumulation of other muscle-specific proteins, one can assume that, when myomesin can be detected in a striated pattern by immunofluorescence, the skeleton of the myofibril is being assembled since the striations are evenly spaced as in sarcomeres.

Structural studies (10, 32) show myomesin and M-protein

to be tightly bound to myosin thick filaments in the region of the M-band under various treatments resulting in myofibrillar dissolution. By contrast, M-CK is more easily released and is not consistently associated with individual thick filaments. These findings implicate myomesin and M-protein as myosin-associated proteins that form a sheath around the thick filament in the M-band region as postulated in previous models (14, 33) with M-CK providing m-bridges at the M4,4' and possibly the M1 substriations.

The temporal appearances of myomesin and M-protein suggest that myomesin plays a role in providing a scaffold for the bipolar orientation of the myosin filaments with a punctate pattern of fluorescence giving way to a striated pattern as adjacent myofibrils become better aligned. The delayed accumulation of M-protein in pectoral muscle, its lower concentration in heart, and its absence from slow fibers of the anterior latissimus dorsi suggest that it is integrated into the M-band at a later stage of differentiation and that its presence may not be crucial for the initial assembly of myofibrils. It may come into play later, perhaps in the "fine tuning" of M-band alignment or in the specialized functions of different fiber types. Examination of myofibrillar assembly at the electron microscopic level with appropriate antibody markers will help to resolve these questions. Further studies at the level of biosynthesis and structure of the M-band protein genes will be necessary to learn more about the roles of these proteins in myofibrillar assembly and function.

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