

A New 185,000-dalton Skeletal Muscle Protein Detected by Monoclonal Antibodies

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ABSTRACT The M line, which transverses the center of the thick filament region of skeletal muscle sarcomeres, appears to be a complex array of multiple structural elements. To date, two proteins have definitely been shown to be associated with the M line. They are MM-CK, localized in the M 4,4' substriations, and a 165,000-dalton (164 kd) protein, referred to as both M-protein and myomesin. Here we report the positive identification of a third M-line protein of 185 kd. In the course of making monoclonal antibodies (mAbs) against a 165-kd fraction, we also obtained mAbs that bound to the M line of isolated myofibrils as detected by indirect immunofluorescence, but recognized a protein band of 185 kd in immunoblotting experiments with either the original immunogen or low ionic strength myofibril extracts as antigenic targets. The evidence that the 185- and 165-kd proteins are distinct protein species is based on the separation of the two proteins into discrete peaks by ion exchange chromatography, the distinctive patterns of their degradation products, and non-cross-reactivity of any of seven mAbs. These mAbs recognize three unique antigenic determinants on the 185-kd molecule and at least two and probably four sites on the 165-kd molecule as determined from competitive binding and immunofluorescence experiments. To resolve the problem of multiple nomenclature for the 165-kd protein, the 185-kd protein will be referred to as myomesin and the 165-kd protein as M-protein.

Myofibrils, the organelles that form the contractile apparatus of cross-striated muscle cells, show distinct structures perpendicular to the thick and thin filaments. The Z-line structure that defines single sarcomeres anchors thin filaments and probably giant proteins such as titin (1) as well. Several possible functions for the other prominent transverse structure, the M line, have been proposed (2–8). More and more Z- and M-line protein components have been detected, isolated, and characterized, which has revealed an ever increasing complexity of these structures. The existence of at least three structural elements has been postulated for the M-line region (9), where high resolution electron microscopy reveals a minimal number of five transverse striations and a so called M filament, which parallels the myosin filaments in the M region (2, 10–13).

To date, two proteins have definitely been shown to be associated with the M line; one is MM-CK (4, 14, 15) and the other is a 165,000-dalton (165-kd) protein (3, 16–18). MM-CK has been assigned to substriations M4 and M4' (19, 20), while the sublocalization of the high molecular weight M-line protein is still unclear. However, from several observations in

our laboratory and in other laboratories as well, it has been concluded that additional protein components must be located within the M-line structure.

Although polyclonal antibodies made against a purified 165-kd M-line protein fraction specifically stained the M line in immunocytochemical tests, depending on preparation and extraction conditions of the antigen, multiple bands were frequently apparent after gel electrophoresis (16, 18) and subsequent immunoblotting (Doetschman, unpublished observations), thus suggesting additional M-line proteins. Indication for the existence of a potential M-line protein of 190 kd found in myofibril extracts had been reported earlier (21), but an unambiguous assignment as an M-line protein had not been made.

Here we report the positive identification of a new 185-kd M-line protein and the resolution of the high molecular weight 185- and 165-kd proteins as two independent M-line-specific components by monoclonal antibodies (mAbs).¹

¹ *Abbreviations used in this paper:* DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; mAbs, monoclonal antibodies; PMSF, phenylmethylsulfonyl fluoride.

MATERIALS AND METHODS

M-line Protein Purification

M-line proteins used for immunization were isolated from pectoralis muscle of adult chicken according to Trinick and Lowey (17) with the modifications applied by Eppenberger and Strehler (22). Additional modifications used in a subsequent purification (Fig. 4) are as follows: (a) Buffer volumes were increased twofold. (b) The initial homogenization and wash buffer [0.1 M KCl, 1 mM EGTA, 5 mM EDTA, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF) (SERVA Feinbiochemica GmbH & Co., Heidelberg, West Germany), pH 7] contained 1 mM L-tosylamide-2-phenylethyl chloromethyl ketone (TPCK) (Sigma Chemical Co., St. Louis, MO), 0.5 mM *N*-p-tosyl-L-lysine chloromethyl ketone (TLCK) (Sigma Chemical Co.), and 0.5% Triton X-100. Stock solutions of the protease inhibitors PMSF, TPCK, and TLCK were dissolved in DMSO. (c) The saturated $(\text{NH}_4)_2\text{SO}_4$ solution used in the 32% ammonium sulfate cut of the high salt extract contained 1 mM MgCl_2 and 0.3 mM EGTA; (d) After dialysis against 1 mM EDTA, 0.3 mM DTT, 10 mM potassium phosphate, pH 6.9, to precipitate actomyosin, ammonium sulfate was added to the supernatant to give a concentration of 50%. The redissolved pellet was dialyzed for 24 h instead of 48 h in three changes of buffer. Aliquots of samples from each stage of the preparation were analyzed for M-line protein content by 7% SDS PAGE followed by Coomassie Blue staining or immunoblotting with mAbs.

Monoclonal Antibodies

BALB/c mice were immunized intraperitoneally with 100 μg of alum-precipitated, purified M-line protein fraction. 3 mo later they were boosted with 100 μg of protein intravenously. After 3 d the spleen cells were isolated and fused (23) with P3-NS1/1Ag4-1 (NS-1) mouse myeloma cells, and clones were grown in Dulbecco's modified Eagle's hypoxanthine/aminopterin/thymidine medium (24) containing 15% selected newborn calf serum. Selected hybridomas were subcloned by limiting dilution.

Solid-phase Immunoassays

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA): An ELISA plate binding assay (25) was used for screening culture supernatants. PVC microtiter plates (Costar, Cambridge, MA) were coated with 1 μg of antigen per well. mAbs were added for 2 h, followed by peroxidase-conjugated rabbit anti-mouse IgG (Nordic Immunological Laboratories, Tillburg, The Netherlands) for 1 h. The bound enzyme was indicated by the addition of the substrates *o*-phenylenediamine and H_2O_2 , and the absorbance was read at 490 nm with a Microelisa Autoreader (Dynatech Laboratories Inc., Alexandria, VA). Background values between 0.02 and 0.04 A U obtained with NS-1 culture supernatants as the primary antibody were subtracted from experimental values.

COMPETITIVE BINDING EXPERIMENTS: Radioactively labeled mAbs were obtained by culturing the hybridomas in methionine-free hybridoma medium containing 1% newborn calf serum and 10 $\mu\text{Ci}/\text{ml}$ [^{35}S]methionine (New England Nuclear, Boston, MA) for 30 h (26). ^{35}S -labeled mAbs were separated from free [^{35}S]methionine by G-25 column chromatography, and 18,000–20,000 dpm were added per well coated with 1 μg of target antigen in the presence of increasing concentrations of unlabeled mAb. After incubations of 3 h or overnight, the bound radioactivity was solubilized in 100 μl of 2 N NaOH and counted in 4 ml of scintillation fluid (Dimilume-30, Packard Instrument Co., Downers Grove, IL). Background values in wells lacking target antigen were ~ 40 dpm/well.

Myofibril Preparation

Fiber bundles were prepared from chicken pectoralis major (15), except that the fiber bundles were kept in solution A (0.1 M KCl, 1 mM EGTA, 5 mM EDTA, 1 mM DTT, pH 7.0) containing 50% (vol/vol) glycerol overnight at 4°C. After a change to fresh buffer, the fiber bundles were stored at -20°C .

Myofibrils were excised from fiber bundles and prepared according to Kundrat and Pepe (27) with the precautions given by Heizmann et al. (28).

Immunofluorescence

A drop of myofibrils was added to a glass coverslip in a 60-mm Petri dish for 1 min, then washed one time quickly by flooding the dish with solution A (~ 3 ml), followed by two 5-min washes in solution A. For SDS treatment, myofibrils were fixed with 3% paraformaldehyde in solution A for 15 min and washed with 0.1 M glycine in solution A once quickly, twice for 5 min each, and once for 15 min. Myofibrils were then exposed to 0.1% SDS in PBS for 2

min, washed three times for 5 min each in PBS followed by 1% Triton X-100 in PBS for 1 min, and again washed in PBS.

For immunofluorescence, 30 μl of mAbs as undiluted culture supernatants were added for 30 min, washed 3 times for 5 min each in PBS followed by rhodamine-conjugated sheep anti-mouse IgG (Cappel Laboratories Inc., Cochranville, PA) (1:500 in PBS) for 30 min, and again washed in PBS. All procedures were carried out at room temperature. Coverslips were mounted on a glass slide in one drop of 0.1 M glycine, 50% glycerol, pH 9.0, for fluorescence microscopy on a Zeiss microscope equipped with epilluminescence and a rhodamine filter pack (Carl Zeiss, Oberkochen, West Germany).

Immunoblotting after SDS PAGE

Samples were electrophoresed on 7% SDS polyacrylamide slab gels with 3% stacking gels (29), and the proteins were electrophoretically transferred to nitrocellulose membranes (30). After blocking nonspecific binding sites with PBS containing 10% fetal calf serum, the nitrocellulose membranes were reacted sequentially with mAbs (undiluted culture supernatants) overnight on a shaker at room temperature and with peroxidase conjugated rabbit anti-mouse IgG (1:300 in PBS with 10% serum) for 4 h also on a shaker at room temperature. After each incubation, nitrocellulose membranes were washed for ~ 30 min in three changes of PBS plus 10% serum. Color development was obtained with a 1:10 dilution of chloronaphthol stock solution (3 mg/ml methanol) in PBS plus 1 $\mu\text{l}/\text{ml}$ 30% H_2O_2 and stopped by a 5-min wash in H_2O . Proteins were visualized in duplicate gels by silver stain (Bio-Rad Laboratories, Richmond, CA).

RESULTS

mAbs were generated to more precisely localize and characterize the 165-kd M-line protein. BALB/c mice were immunized with a 165-kd protein fraction purified from chicken pectoralis major (see Materials and Methods) and the spleen cells were fused with NS-1 mouse myeloma cells. After initial selection by ELISA screening using the immunogen as the target antigen, seven mAbs were obtained that specifically bound to the M-line of isolated chicken myofibrils as demonstrated by indirect immunofluorescence. Four of the mAbs, B5 (Fig. 1a), A2, B4, and C2, bound readily to the M line of native myofibrils. However, binding of A6 (Fig. 1, c and d) as well as A1 and A5 was detectable only after treatment of

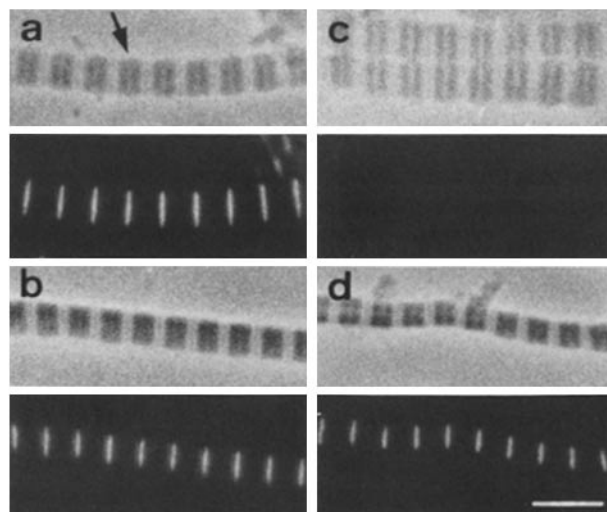


FIGURE 1 Reactivity of monoclonal antibodies B5 and A6 with glycerinated myofibrils with and without SDS treatment detected by indirect immunofluorescence. B5 binding is localized in the M-line region (arrow) of both native myofibrils (a) and SDS-treated myofibrils (b). A6 does not bind to native myofibrils (c), whereas M-line fluorescence is detectable after SDS treatment (d). Phase-contrast (top) and fluorescence (bottom) images are paired. Bar, 5 μm . $\times 1,900$.

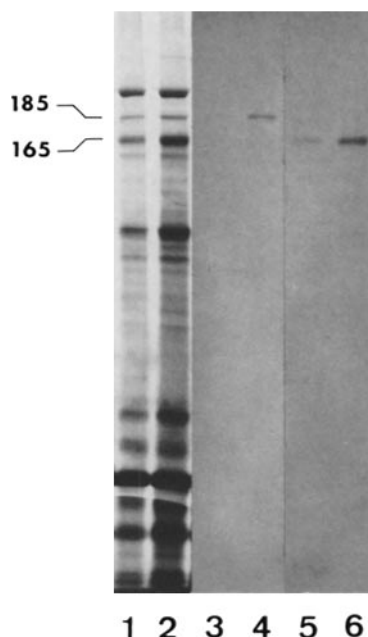


FIGURE 2 Immunoblots of monoclonal antibodies B5 and A6 with myofibril extracts. Glycerinated myofibrils were extracted with 5 mM Tris, 1 mM DTT, pH 7.7, for 1 or 24 h and 25 μ l of each extract was resolved by 7% SDS PAGE. Lanes 1, 3, and 5 contain 1-h extracts; lanes 2, 4, and 6 contain 24-h extracts. Silver stain of 1- and 24-h extracts are shown in lanes 1 and 2, respectively. Extracts in duplicate gels were electrophoretically transferred to nitrocellulose membrane and reacted with B5 (lanes 3 and 4) and A6 (lanes 5 and 6).

Standards for molecular weight estimations included fibronectin (230- and 220-kD subunits) glycogen debranching enzyme (165 kD), phosphorylase *b* (94 kD), BSA (57 kD), and ovalbumin (45 kD). The apparent molecular weights of the proteins reacting with mAbs are 185,000 (lanes 3 and 4) and 165,000 (lanes 5 and 6).

the myofibrils with 0.1% SDS. It is possible that SDS treatment affects the conformation of the antigen or the accessibility of the antigenic determinant within the structure of the myofibril. Since SDS treatment did not affect the binding of mAbs that recognize antigenic determinants in native myofibrils (B5 in Fig. 1*b*), subsequent immunocytological localization experiments were performed on SDS-treated myofibrils.

The molecular specificity of binding was demonstrated on immunoblots of myofibril extracts. Extracts were prepared by suspending isolated myofibrils in low ionic strength buffer (5 mM Tris, 1 mM DTT, pH 7.7) (18) for 1 h or 24 h and the extracted proteins were resolved by 7% SDS PAGE. In Fig. 2, lanes 1, 3, and 5 contain 1-h extracts and lanes 2, 4, and 6 contain 24-h extracts. Fig. 2, lanes 1 and 2, demonstrate the silver stain pattern of total extracted protein. Proteins resolved on duplicate gels were electrophoretically transferred to nitrocellulose membranes and reacted with mAbs. Two patterns of staining emerged. One group of mAbs (A1, A5, A6, and C2) represented here by A6 binds to the 165-kD protein (Fig. 2, lanes 5 and 6) which has been previously localized in the M line (3, 16–18, 31). However, another group of mAbs (A2, B4, B5) represented here by B5 reacts with a distinct band of 185-kD (Fig. 2, lanes 3 and 4). Based on immunofluorescence localization of this group of mAbs within the myofibril (B5 shown in Fig. 1, *a* and *b*), the 185-kD protein appears to be an M-line component that has not been previously identified. Both proteins are released in increasing amounts up to 24 h of myofibril extraction; however, the relative intensities of the silver-stained bands (Fig. 2, lanes 1 and 2) indicate that less of the 185-kD protein is present compared with the 165-kD protein. While this difference could represent quantitative differences in myofibril content, it could also be due to differential extraction properties of the two proteins.

Differentially extracted myofibrils from the experiment shown in Fig. 2 were assayed for the presence of the 185- and

165-kD proteins by indirect immunofluorescence. After 1 h (data not shown) and to a greater extent after 24 h of extraction (Fig. 3), a reduced intensity of M-line fluorescence was observed with both mAb B5 against the 185-kD protein (Fig. 3*b*) and mAb A6 against the 165-kD protein (Fig. 3*d*). The binding patterns of mAbs B5 and A6 to extracted myofibrils provides additional evidence for the specific extraction of the 185- and 165-kD proteins, respectively, from the M line.

Since there was considerable evidence from immunoblotting experiments that our original immunogen contained two distinct M-line proteins of 185 and 165 kD, we attempted to separate and purify these two proteins. Owing to the susceptibility of myofibrillar proteins to degradation by endogenous proteases (3, 7, 17, 21, 32–35) the purification procedure was repeated with the following additional precautions. Protease inhibitors TPCK, TLCK, and PMSF (see Materials and Methods) were added to the initial homogenization and wash buffer containing 0.5% Triton X-100. Purification was performed in the presence of PMSF according to the procedure of Eppenberger and Strehler (22) with the modifications indicated in Materials and Methods. The resulting elution profile of the DEAE cellulose (DE 52, Whatman Ltd., Maidstone, England) column is shown in Fig. 4. Coomassie Blue-stained 7% SDS gels of corresponding fractions are inserted above the elution profile. Two proteins, most likely glycogen debranching enzyme (165 kD) and phosphorylase *b* (94 kD) (17, 36), are eluted in fractions 62–72 and 80–137, respectively. In contrast to previous purifications where only one additional broad peak could be resolved, two peaks appear around fraction 96 and fraction 113. The first of the two peaks contains the 185-kD protein as well as several proteins with slightly lower molecular weights as shown in the gel patterns. The second peak contains the 165-kD M-line protein as well

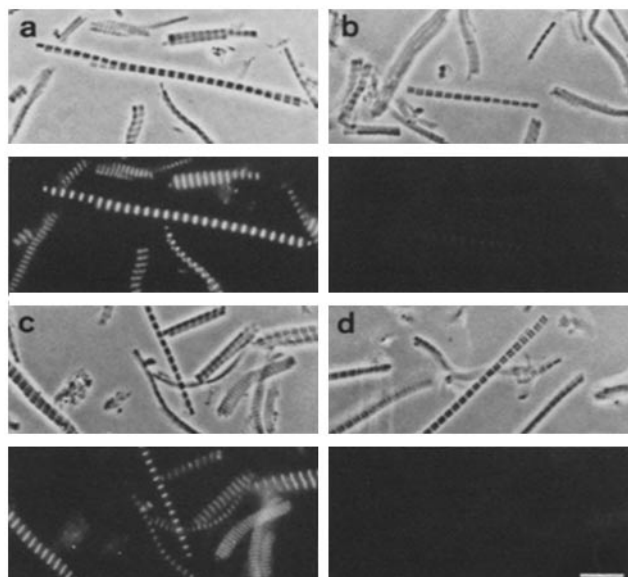


FIGURE 3 Reactivity of mAb B5 against the 185-kD protein and mAb A6 against the 165-kD protein with nonextracted (*left*) and extracted (*right*) glycerinated myofibrils detected by indirect immunofluorescence. B5 was reacted with isolated control myofibrils in solution A (*a*) and 24-h extracted myofibrils from experiment in Fig. 2 (*b*). A6 was reacted with control myofibrils (*c*) and 24-h extracted myofibrils (*d*). Paired photographs are phase contrast (*top*) and fluorescence (*bottom*). All myofibrils were SDS treated prior to reaction with mAbs. Bar, 10 μ m. \times 600.

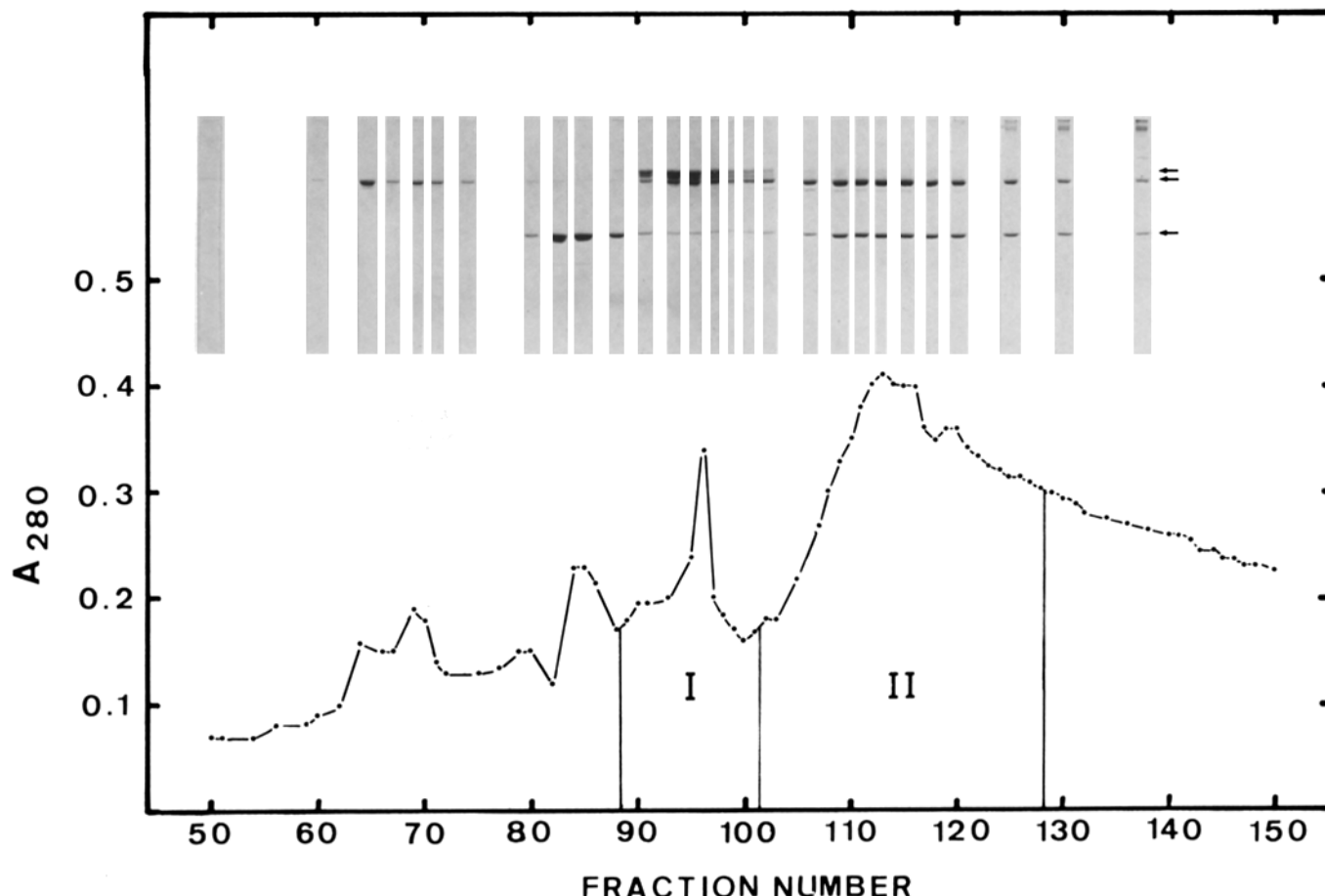


FIGURE 4 DE 52 column chromatography profile of high salt extract of pectoralis muscle. Actomyosin was removed from a crude high salt extract by precipitation under low salt conditions (see Materials and Methods) and the remaining extract was eluted from a DE 52 column with a linear KCl gradient (0–0.3 M KCl). Insets are 7% SDS polyacrylamide gels of aliquots from corresponding column fractions stained with Coomassie Blue. Arrows to the right indicate positions of 185-, 165-, and 94-kd protein bands. The first minor peaks contain debranching enzyme (165 kd) followed by phosphorylase *b* (94 kd) which persists in its inactive form throughout the remainder of the fractions. The two major peaks contain the 185-kd protein and the 165-kd protein, respectively. Fractions 89–101 (I) and 102–128 (II) were pooled and phosphorylase *b* was removed by Sepharose-AMP column chromatography.

as the 94-kd phosphorylase *b*. These two peaks were pooled separately as fractions I and II and the phosphorylase *b* was removed by adsorption to a Sepharose-AMP column.

The selective specificity of the mAbs for one or the other of these two fractions was demonstrated in an ELISA assay using both fraction I and fraction II as target antigens (Table I). The ratios of fraction II to fraction I binding indicate that one group of mAbs (B2, B4, and B5) preferentially binds to fraction I containing the 185-kd and the other high molecular weight bands, while a second group (A1, A5, and C2) including A6 (Fig. 2) binds predominantly to fraction II that contains primarily the 165-kd protein. The small amount of binding obtained with the alternate target antigen is due to slight cross-contamination of the two fractions. From these data and the differential staining patterns obtained in immunoblotting experiments, we infer the existence of two M-line proteins with distinct biochemical and immunological properties.

To further characterize the two M-line protein species, the susceptibility of both the 185- and 165-kd proteins to proteolysis by endogenous protease is illustrated in Fig. 5. Myofibrils were extracted for 24 h (Materials and Methods; Fig. 2) and extracts were kept at 4°C for an additional 24 h. Proteins

TABLE I
ELISA Assay for Reactivity of Monoclonal Antibodies with M-line Protein Fractions I and II

Monoclonal antibody	Pooled fractions from DE 52 column*		Ratio of reactivities II/I
	I	II	
	<i>A₄₉₀</i>		
A2	0.42	0.03	0.07
B4	0.72	0.13	0.18
B5	1.22	0.44	0.36
A1	0.25	0.99	3.9
A5	0.47	1.19	2.7
C2	0.14	0.95	6.9

* Pooled fractions described in Fig. 4.

from the extract were resolved on 7% SDS polyacrylamide gels, transferred to nitrocellulose, and reacted with mAbs. Fig. 5, lanes 1 and 2 (in duplicate) show the reaction of B5, a mAb that recognizes the 185-kd protein, with the myofibril extract. In addition to the 185-kd band, B5 stains several bands between 185 and 165 kd that are also detected in fraction I from the DE 52 column (Fig. 4) plus a band immediately

below the 165-kd band. Fig. 5, lanes 3 and 4 (in duplicate) show the reaction of A6, a mAb that recognizes the 165-kd protein. A6 detects a series of bands of molecular weight <165,000 as well as a band of considerably higher molecular weight. This higher molecular weight band has been seen previously (17) and is thought to be an aggregate. The bands toward the bottom of the blot probably represent small molecular weight degradation products that are not well resolved. The distinctive patterns of degradation products obtained in this analysis are further evidence that the 185- and 165-kd bands represent different molecular entities. The lack of degradation in the initial low ionic strength extracts (Fig. 2) may have been due to prior storage of the stretched muscle fibers in solution A containing 50% glycerol which has been reported to inhibit endogenous protease activity (21). However, prolonged maintenance at 4°C resulted in considerable degradation.

The next step in the characterization of the two high molecular weight M-line proteins was to determine how many antigenic determinants on each of the proteins were detected by the mAbs. To this end, competitive binding experiments were performed by adding each of the ³⁵S-labeled mAbs in the presence of increasing concentrations of unlabeled mAb to a target containing both the 185- and 165-kd proteins. Fig. 6 shows representative competition curves for one of the mAbs against each of the M-line proteins. In Figure 6A, unlabeled B5 displaces 91% of the binding of ³⁵S-labeled B5 to the 185-kd protein. Neither the other mAbs against the 185-kd protein (A2 and B4) nor the mAbs against the 165-kd protein (A5 and A6) or NS-1 culture supernatant used here

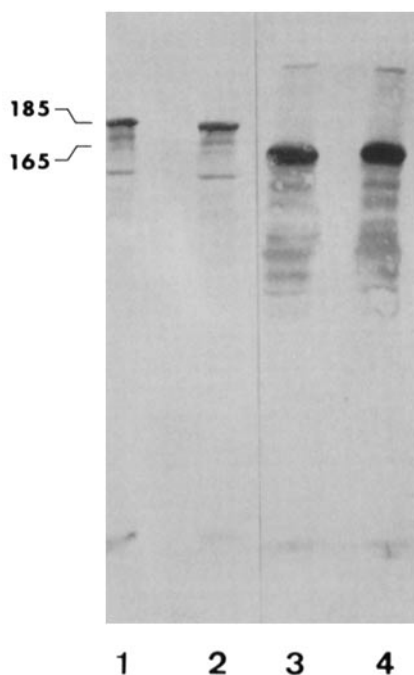


FIGURE 5 Proteolytic patterns of 185- and 165-kd M-line proteins. Myofibril extracts prepared from glycerinated myofibrils by the same procedure as those in Fig. 2 were kept at 4°C for 24 h. Approximately 45 μ g of myofibril extract in 4 \times sample buffer was loaded in each lane. After 7% SDS PAGE and transfer to nitrocellulose membranes, the extracts in lanes 1 and 2 were immunoblotted with mAb B5 against the 185-kd protein. Duplicate extracts in lanes 3 and 4 were immunoblotted with mAb A6 against the 165-kd protein. Apparent molecular weights were determined as in Fig. 2.

as negative controls demonstrated significant competition. This result indicates that B5 recognizes a unique antigenic determinant on the 185-kd molecule. Similar results were obtained for the other mAbs against the 185-kd protein (A2 and B4), indicating the presence of two additional unique antigenic determinants characterized by A2 and B4.

Similar experiments were carried out for mAbs recognizing the 165-kd antigen. The competition for ³⁵S-labeled A6 binding is shown in Fig. 6B. Both unlabeled A6 and A1 compete with ³⁵S-labeled A6 for binding to the 165-kd protein, although A1 competes to a lesser extent (44% compared with 87% for A6). This discrepancy in maximum competition for binding could be due to a lower binding affinity for A1 since in the reciprocal competition experiment unlabeled A6 competes with ³⁵S-labeled A1 equally as well as unlabeled A1 (98% compared with 85%, respectively) with similar competitive binding curves. While it is apparent that neither the other mAbs against the 165-kd protein (A5 and C2) nor the negative controls (mAb B4 against the 185-kd protein and NS-1 culture supernatant) compete specifically for ³⁵S-labeled A6 binding, the slight slope of the curves for these unlabeled antibodies at the highest concentration suggests nonspecific interference of binding. In competitive binding experiments with the other mAbs against the 165-kd protein, A5 and C2 also demonstrate competitive inhibition of binding. The inhibition of ³⁵S-labeled mAb binding by negative controls is most evident when the maximum binding of labeled mAb in the absence of unlabeled competitors falls below 600 dpm, suggesting that the mAbs with lower binding affinities are more sensitive to nonspecific binding interference.

Table II summarizes the results of the competitive binding experiments for all the mAbs against both M-line proteins. All the mAbs against the 185-kd protein recognize distinct determinants, while 2 sets of mAbs (A5 and C2, A6 and A1) against the 165-kd protein demonstrate competitive binding suggesting identical or overlapping antigenic determinants or steric hindrance of binding. The finding that C2 binds to native myofibrils while A5 binds only to SDS-treated myofibrils, in addition to the evidence that A5 and A6, but not C2 or A1, bind to myofibrils from muscles of other species (Dr. Monika Eppenberger, this laboratory, personal communication), suggests that the antigenic determinants for these two sets of mAbs are not identical. Thus we have produced mAbs against three antigenic determinants on the 185-kd M-line protein as well as mAbs against at least two and probably against four determinants on the 165-kd M-line protein.

DISCUSSION

Biochemical characterization of high molecular weight M-line proteins has been fraught with difficulties owing to contamination and degradation of the proteins during purification (7, 16–18). Polyclonal antibodies were raised against the most purified high molecular weight M-line protein fraction (165 kd) and these antibodies also reflected the heterogeneous nature of the proteins in this purified fraction but were unable to discriminate between 165-kd degradation products and the presence of additional proteins. These problems were circumvented by generating mAbs directed against the M-line protein fraction.

We have obtained mAbs that recognize two distinct M-line proteins with molecular weights of 165,000 and 185,000. The characterization of these mAbs indicates that all the mAbs recognize unique or, in two instances, possibly overlapping

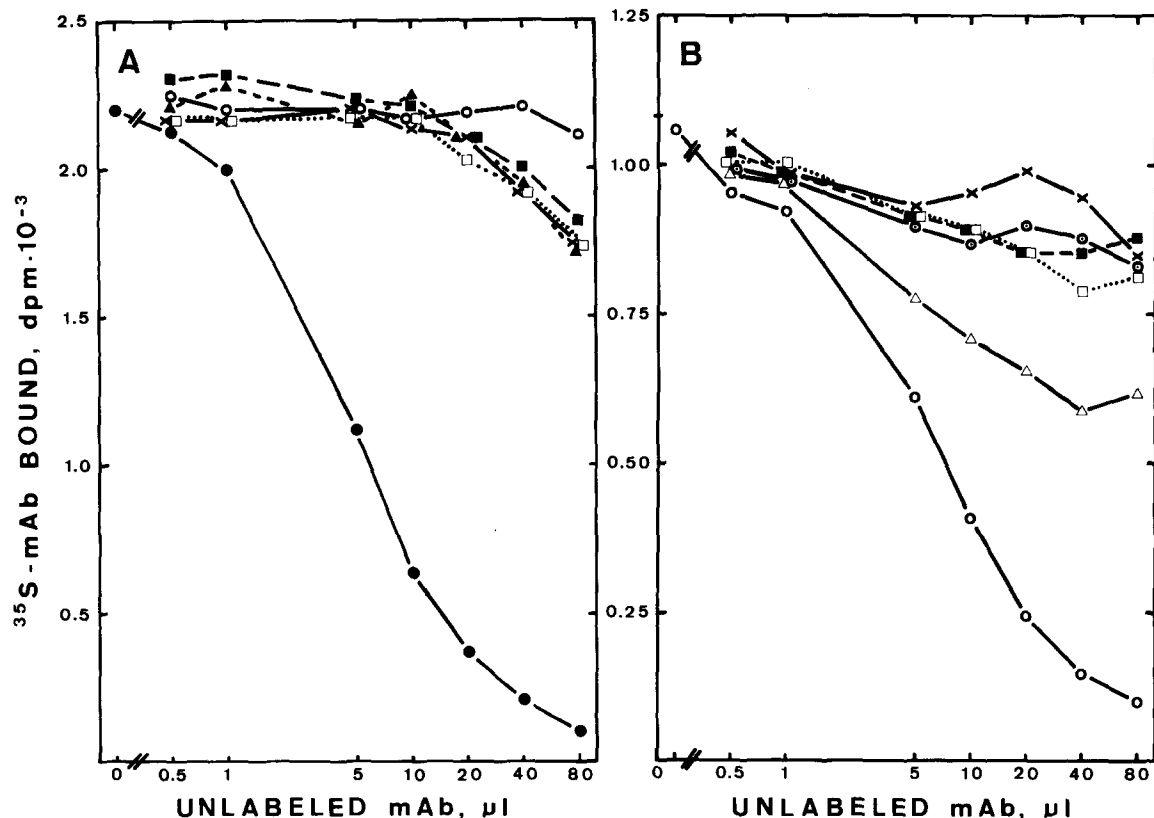


FIGURE 6 Competitive binding curves for the mAb B5 against the 185-kd protein (A) and mAb A6 against the 165-kd protein (B). (A) ^{35}S -labeled B5 (18,000 dpm/well) binding in the presence of increasing concentrations of B5 (●), A6 (○), B4 (■), NS-1 supernatant (×), A5 (□), or A2 (▲). The target antigen was purified fraction I at 1 $\mu\text{g}/\text{well}$. (B) ^{35}S -labeled A6 (17,000 dpm/well) binding in the presence of increasing concentrations of A6 (○), A1 (Δ), B4 (■), NS-1 supernatant (×), C2 (◊), or A5 (□). The original 165-kd immunogen, which also contained the 185-kd protein, was used as the target antigen at 1 $\mu\text{g}/\text{ml}$. In both experiments antibodies were reacted with the targets for 3 h. The bound radioactivity was solubilized in 100 μl of 2 N NaOH and counted. Background values obtained from wells not coated with target were subtracted from the experimental values.

TABLE II
Specificity of Monoclonal Antibodies for Antigenic Determinants

Unlabeled mAbs		^{35}S -labeled mAbs						
		Anti-185-kd protein			Anti-165-kd protein			
		A2	B4	B5	A1	A6	A5	C2
Anti-185-kd protein	A2	+	—	—	—	ND*	ND	—
	B4	—	+	—	—	—	—	—
	B5	—	—	+	—	ND	ND	—
Anti-165-kd protein	A1	—	—	ND	+	+	—	—
	A6	—	—	—	+	+	—	—
	A5	—	—	—	—	—	+	+
	C2	ND	ND	ND	—	—	+	+
Control	NS-1	—	—	—	—	—	—	—

* ND, not determined.

antigenic determinants and that none of the mAbs reacting with the 185-kd protein demonstrates cross-reactivity with the 165-kd species and vice versa. The 165-kd M-line protein has been previously purified and characterized (3, 17, 22). While other high molecular weight myofibrillar proteins have been reported (21, 37), their localization could only be inferred by circumstantial evidence. The finding of the 185-kd protein reported here is to our knowledge the first concrete evidence for a second high molecular weight M-line protein. Inability to detect the 185-kd protein in previous purifications

of the 165-kd protein may have been due to the small quantity of this protein present in myofibrils relative to the 165-kd protein, degradation, poor resolution on overloaded polyacrylamide gels, or a combination of these effects.

Polyclonal antibodies raised in rabbits against a chicken 165-kd M-line protein fraction were used to detect the appearance of M lines during myofibrillogenesis in cultured cells (31) and were shown to react specifically with skeletal muscle and heart in a variety of species (31, 38). In immunoblot experiments, these polyclonal antibodies detect all the bands

recognized by mAbs against both the 165- and the 185-kd protein. Work in progress using the mAbs described here indicates that the protein that had been detected by the polyclonal antibodies in short-term cell cultures (31) and in early chick embryos (39) is most likely the 185-kd M-line protein and not the 165-kd protein as previously believed. Since myomesin was the name given to the M-line protein appearing early in myogenesis (31), we propose, in agreement with Dr. T. Masaki (U. of Tsukuba, Japan, personal communication), that "myomesin" be used to designate the 185-kd M-line protein. The 165-kd protein will continue to be called "M-protein" (3).

The inability of the polyclonal antibodies to clearly define the localization of the 165-kd protein within the M line (19) may be due to the heterogeneity of these polyclonal antibodies. Experiments are now in progress to determine the substructural localization of both the 185-kd myomesin and the 165-kd M protein within the M line using mAbs. The selective mAb probes will also be used to better define and extend the initial findings on the developmental regulation of these two proteins during myogenesis in vivo and in cell culture.

We wish to thank Barbara Niederöst for her enthusiastic technical assistance, Dr. Theo Wallimann for his helpful discussions and reading of the manuscript, and Mrs. DeGiorgi, Mrs. Leuzinger, and Ms. Abächerli for their skillful preparation of the manuscript.

This work was supported by a Swiss National Science Foundation grant (No. 3.707-0.80) and by a grant from the Muscular Dystrophy Association.

Received for publication 31 May 1983, and in revised form 22 August 1983.

REFERENCES

- Wang, K., and R. Ramirez-Mitchell. 1983. A network of transverse and longitudinal intermediate filaments is associated with sarcomeres of adult vertebrate skeletal muscle. *J. Cell Biol.* 96:562-570.
- Knappes, G. G., and F. Carlsen. 1968. The ultrastructure of the M line in skeletal muscle. *J. Cell Biol.* 38:202-211.
- Masaki, T., and O. Takaiti. 1974. M-Protein. *J. Biochem.* 75:367-380.
- Wallimann T., D. C. Turner, and H. M. Eppenberger. 1977. Localization of creatine kinase isoenzymes in myofibrils. I. Chicken skeletal muscle. *J. Cell Biol.* 75:297-317.
- Mani, R. S., and C. M. Kay. 1978. Interaction studies of the 165,000 dalton protein component of the M-line with the S2 subfragment of myosin. *Biochim. Biophys. Acta.* 536:134-141.
- Wallimann, T., T. Schlösser, and H. M. Eppenberger. 1982. ATP-regeneration potential of M-line bound creatine kinase. Physiological significance. *J. Muscle Res. Cell Motil.* 3:503-504.
- Woodhead, J. L., and S. Lowey. 1982. Size and shape of skeletal muscle M-protein. *J. Mol. Biol.* 157:149-154.
- Eppenberger, H. M., J. C. Perriard, and T. Wallimann. 1983. Analysis of creatine kinase isozymes during muscle differentiation. In *Isozymes: Current Topics in Biological and Medical Research*. Alan R. Liss, Inc., New York. 7:19-37.
- Luther, P., and J. Squire. 1978. Three-dimensional structure of the vertebrate muscle M-region. *J. Mol. Biol.* 125:313-324.
- Pepe, F. 1972. The myosin filament: immunochemical and ultrastructural approaches to molecular organization. *Cold Spring Harbor Symp. Quant. Biol.* 37:97-108.
- Sjöström, M., and J. M. Squire. 1977. Fine structure of the A-band in cryo-sections. *J. Mol. Biol.* 109:49-68.
- Sjöström, M., and J. M. Squire. 1977. Cryo-ultramicrotomy and myofibrillar fine structure: a review. *J. Microsc. (Oxf.)* 11:239-278.
- Luther, P. K., P. M. G. Munro, and J. M. Squire. 1981. Three-dimensional structure of the vertebrate muscle A-band. III. M-region structure and myosin filament symmetry. *J. Mol. Biol.* 151:703-730.
- Turner, D. C., T. Wallimann, and Hans M. Eppenberger. 1973. A protein that binds specifically to the M-line of skeletal muscle is identified as the muscle form of creatine kinase. *Proc. Natl. Acad. Sci. U. S. A.* 70:702-705.
- Wallimann, T., G. Pelloni, D. Turner, and H. Eppenberger. 1978. Monovalent antibodies against MM-creatine kinase remove the M-line from myofibrils. *Proc. Natl. Acad. Sci. USA* 75:4296-4300.
- Palmer, E. G. 1975. Antibody localization studies of the M-line in striated muscle. *Can. J. Zool.* 53:788-799.
- Trinick, J., and S. Lowey. 1977. M-protein from chicken pectoralis muscle: isolation and characterization. *J. Mol. Biol.* 113:343-368.
- Strehler, E., G. Pelloni, C. Heizmann, and H. Eppenberger. 1980. Biochemical and ultrastructural aspects of M, 165,000 M-protein in cross-striated chicken muscle. *J. Cell Biol.* 86:775-783.
- Strehler, E., E. Carlson, H. M. Eppenberger, and L. E. Thornell. 1983. Ultrastructural localization of M-band proteins in chicken breast muscle as revealed by combined immunocytochemistry and ultramicrotomy. *J. Mol. Biol.* 166:141-158.
- Wallimann, T., T. C. Doetschman, and H. M. Eppenberger. 1983. Novel staining pattern of skeletal muscle M-lines upon incubation with antibodies against MM-creatine kinase. *J. Cell Biol.* 96:1772-1779.
- Etlinger, J. D., R. Zak, and D. A. Fischman. 1976. Compositional studies of myofibrils from rabbit striated muscle. *J. Cell Biol.* 68:123-141.
- Eppenberger, H. M., and E. E. Strehler. 1982. Preparation and properties of M-line proteins. *Methods Enzymol.* 85:139-149.
- Galfre, G., and C. Milstein. 1981. Preparation of monoclonal antibodies: strategies and procedures. *Methods Enzymol.* 73:3-46.
- Kennett, R. H., K. A. Denis, A. S. Tung, and N. R. Klinman. 1978. Hybrid plasmacytoma production: fusion with adult spleen cells, monoclonal spleen fragments, neonatal spleen cells and human lymphocytes. *Curr. Top. Microbiol. Immunol.* 81:77-93.
- Voller, A., D. E. Bidwell, and A. Bartlett. 1979. The enzyme linked immunosorbent assay (ELISA). Dynatech Europe, Guernsey, Great Britain.
- de St. Groth, S. F., and D. Scheidegger. 1980. Production of monoclonal antibodies: strategy and tactics. *J. Immunol. Methods.* 35:1-21.
- Kundrat, E., and F. A. Pepe. 1971. The M band: studies with fluorescent antibody staining. *J. Cell Biol.* 48:340-347.
- Heizmann, C. W., and H. M. Eppenberger. 1978. Isolation and characterization of glycogen phosphorylase b from chicken breast muscle: comparison with a protein extracted from the M-line. *J. Biol. Chem.* 253:270-273.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 227:680-685.
- Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of protein from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* 76:4350-4353.
- Eppenberger, H., J. C. Perriard, U. Rosenberg, and E. Strehler. 1981. The M, 165,000 M-protein myomesin: a specific protein of cross-striated muscle cells. *J. Cell Biol.* 89:185-193.
- Drabikowski, W., A. Gorecka, and A. Jakubiec-Puka. 1977. Endogenous proteinases in vertebrate skeletal muscle. *Int. J. Biochem.* 8:61-71.
- Dayton, W., J. Schollmeyer, R. Lepley, and L. Cortés. 1981. A calcium-activated neutral protease possibly involved in myofibrillar protein turnover. *Biochim. Biophys. Acta.* 659:48-61.
- Kaur, H., and B. Sanwal. 1981. Regulation of the activity of a calcium-activated neutral protease during differentiation of skeletal myoblasts. *Can. J. Biochem.* 59:743-747.
- Granger, B. L., E. A. Repasky, and E. Lazarides. 1982. Synemin and vimentin are components of intermediate filaments in avian erythrocytes. *J. Cell Biol.* 92:299-312.
- Heizman, C. W., and H. M. Eppenberger. 1979. Glycogen debranching enzyme from chicken pectoralis muscle. Comparison with a 165,000 molecular weight myofibrillar protein. *FEBS Fed. Eur. Biochem. Soc. Lett.* 105:35-39.
- Porzio, M. A., A. M. Pearson, and D. P. Cornforth. 1979. M-line protein: presence of two nonequivalent high molecular weight components. *Meat Science.* 3:31-41.
- Eppenberger, H. M., M. Bähler, T. C. Doetschman, M. Eppenberger, J. C. Perriard, D. Studer, T. Wallimann, and E. E. Strehler. 1982. The M-protein myomesin in cross-striated muscle cells during myofibrillogenesis. In *Muscle Development: Molecular and Cellular Control*. M. L. Pearson and H. F. Epstein, editor. Cold Spring Harbor Lab., Cold Spring Harbor, NY. 429-437.
- Chiquet, M., H. M. Eppenberger, and D. C. Turner. 1981. Muscle morphogenesis: evidence for an organizing function of exogenous fibronectin. *Dev. Biol.* 88:220-235.